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## Select G-protein coupled receptors modulate agonist-induced signaling via a ROCK, LIMK and $\beta$ -arrestin 1 pathway

Nitish Mittal<sup>1,\*</sup>, Kristofer Roberts<sup>1,\*</sup>, Katsuri Pal<sup>2</sup>, Laurent A. Bentolila<sup>3,6</sup>, Elissa Fultz<sup>1</sup>, Ani Minasyan<sup>1</sup>, Catherine Cahill<sup>4</sup>, Amynah Pradhan<sup>1</sup>, David Conner<sup>5</sup>, Kathryn DeFea<sup>2</sup>, Christopher Evans<sup>1</sup>, and Wendy Walwyn<sup>1</sup>

<sup>1</sup>Dept. of Psychiatry and Biobehavioral Sciences, Stefan Hatos Center for Neuropharmacology, Semel Institute, UCLA, CA

<sup>2</sup>Div. of Biomedical Sciences; Cell, Molecular, and Developmental Biology Program, UCR, CA

<sup>3</sup>Dept. of Chemistry and Biochemistry, UCLA, CA

<sup>4</sup>Dept. of Anesthesia and Perioperative Care, UCI

<sup>5</sup>Dept. of Genetics, Harvard Medical School, Boston, MA

<sup>6</sup>California NanoSystems Institute, UCLA, CA

### Abstract

G-protein coupled receptors (GPCRs) are typically present in a basal, inactive state, but when bound to agonist they activate downstream signaling cascades. In studying arrestin regulation of opioid receptors in dorsal root ganglia (DRG) neurons, we find that agonists of delta opioid receptors ( $\delta$ ORs) activate cofilin through Rho-associated coiled-coiled containing protein kinase (ROCK), LIM domain kinase (LIMK) and  $\beta$ -arrestin 1 ( $\beta$ -arr1), to regulate actin polymerization. This controls receptor function, as assessed by agonist-induced inhibition of voltage-dependent Ca<sup>2+</sup> channels in DRGs. Agonists of opioid-receptor like receptors (ORL1) similarly influence the function of this receptor through ROCK, LIMK and  $\beta$ -arr1. Functional evidence of this cascade was demonstrated in vivo where the behavioral effects of  $\delta$ OR or ORL1 agonists were enhanced in the absence of  $\beta$ -arr1 or prevented by inhibiting ROCK. This pathway allows  $\delta$ OR and ORL1 agonists to rapidly regulate receptor function.

### Introduction

The neuronal cytoskeleton provides both the 3-dimensional structural stability critical for cellular function, and the infrastructure that allows the cell to respond to external stimuli. Dynamic remodeling of the cytoskeleton, particularly of the actin filaments, provides the network along which intracellular proteins may be trafficked as needed. This mechanism is important to shuttle proteins to and from the synapse, as well as allow the Golgi apparatus to sort and traffic newly synthesized proteins to the cell membrane (Lowe, 2011; Salvarezza et al., 2009). Proteins bound for the cell membrane from the Golgi may either be constitutively

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Corresponding author: Wendy Walwyn, Ph.D., UCLA-Semel Institute, Box 77, 760 Westwood Plaza, Los Angeles, CA, 90095,

wwalwyn@ucla.edu.

\*co first-author

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exported through a specific pathway with specialized secretory vesicles that are formed and move continuously along the microtubules, or secreted on demand. A dynamic actin cytoskeleton plays an important role in this process; both the actin severing protein, cofilin, and the upstream kinase, LIMK, have been shown to inhibit or facilitate the release of specific proteins from the Golgi to the cell membrane (Egea et al., 2006; Heimann et al., 1999; Salvarezza et al., 2009).

Similar to many other membrane proteins, newly synthesized G-protein coupled receptors (GPCRs) are sorted, processed, degraded or trafficked from the endoplasmic reticulum to the Golgi. Thereafter, GPCR transfer through the Golgi stack is complex and may involve several escort or chaperone proteins to release the receptor to the cell membrane. For the most part, the rate of GPCR release to the cell membrane is determined by the quality control mechanisms within the ER and once within the Golgi, GPCRs are presumed to be constitutively released to the cell membrane. By regulating the number of GPCRs released to the cell membrane, this biosynthetic pathway is able to influence receptor availability and signaling (Achour et al., 2008; Dong et al., 2007).

$\beta$ -arrestin 1 or 2 recruitment to the protease-activated receptor 2 (PAR<sub>2</sub>), regulates chemotaxis by binding with and activating cofilin and associated regulatory proteins. This alters actin turnover within the leading and trailing edges and allows the cell to migrate as needed (DeFea, 2007; Xiao et al., 2010; Zoudilova et al., 2007; Zoudilova et al., 2010). In examining  $\beta$ -arrestin 1 ( $\beta$ -arr1) regulation of GPCR function in dorsal root ganglia (DRG) neurons, we have found that this arrestin isoform influences the stability of the actin cytoskeleton to rapidly control the magnitude of GPCR signaling.

## Results

The primary afferent neurons of the DRGs, the first order neurons of the analgesic pathway, relay nociceptive information from the peripheral to the central nervous system. In addition to a number of  $G_{i/o}$  coupled GPCRs, these neurons also express the non-visual arrestins (Komori et al., 1999). Of the two isoforms,  $\beta$ -arrestin 2 is known to regulate the function of some  $G_{i/o}$  coupled GPCRs in DRG neurons (Tan et al., 2009; Walwyn et al., 2007). However, whether  $\beta$ -arrestin 1 regulates the function of these GPCRs has not been established.

**$\beta$ -arr1 modulates VDCC inhibition by some GPCRs**—As agonists of  $G_{i/o}$ -coupled GPCRs can inhibit voltage-dependent  $Ca^{2+}$  channels (VDCC), the whole cell patch clamp technique was used to assess  $\beta$ -arr1 regulation of  $G_{i/o}$ -coupled GPCRs in DRG neurons. Of the 5 GPCRs examined, we found that VDCC inhibition by the  $\delta$ OR agonist, DPDPE ( $p < 0.05$  vs  $\beta$ -arr1+/+), and the ORL1 agonist, nociceptin ( $p < 0.001$  vs  $\beta$ -arr1+/+), was enhanced in cells lacking  $\beta$ -arr1. On the other hand, VDCC-inhibition by agonists of the mu opioid, GABA<sub>B</sub> and  $\alpha_{2A}$  adrenergic receptors was not altered (Fig 1A). Further analysis of the  $\delta$ OR-VDCC profile showed that, in addition to the peptidergic agonist DPDPE, the non-peptidergic and arguably more specific  $\delta$ OR agonist, SNC80 (1 $\mu$ M) also resulted in greater VDCC inhibition in medium-sized, but not small, DRG neurons lacking  $\beta$ -arr1 ( $p < 0.001$  vs  $\beta$ -arr1+/+, Fig. 1B). The concentration response curve of SNC80 in medium-sized neurons demonstrate enhanced efficacy and potency of this  $\delta$ OR agonist in  $\beta$ -arr1<sup>-/-</sup> neurons (E<sub>max</sub>;  $\beta$ -arr1+/+; 32.1 $\pm$ 3.1,  $\beta$ -arr1<sup>-/-</sup>; 47.7 $\pm$ 2.5 % inhibition,  $p < 0.001$ , Fig. 1C). This enhanced inhibition was receptor-specific as it was reversed by the delta antagonist naltrindole, (NTI; SNC80 and NTI applied at 1 $\mu$ M;  $\beta$ -arr1+/+; 0.1 $\pm$ 0.6 %,  $\beta$ -arr1<sup>-/-</sup>; 1.1 $\pm$ 1.8 % inhibition). It was also reversed by the ORL1 antagonist, JTC801 (JTC801 and nociceptin at 1 $\mu$ M;  $\beta$ -arr1+/+; 1.6 $\pm$ 7.1 %,  $\beta$ -arr1<sup>-/-</sup>; 6.3 $\pm$ 5.1 %). Medium-sized neurons from different aged mice or of different backgrounds showed the same profile of enhanced

inhibition in  $\beta$ -arr1<sup>-/-</sup> neurons ( $p < 0.05$  vs  $\beta$ -arr1<sup>+/+</sup>, Fig 1D, 1E) which could not be explained by basal changes in Ca<sup>2+</sup> channel density, the relative proportion of N, P/Q and L type Ca<sup>2+</sup> channels present or constitutive  $\delta$ OR-VDCC coupling (SFig. 1).

**$\beta$ -arr1 regulation of  $\delta$ OR desensitization**—As  $\delta$ ORs recruit  $\beta$ -arr1 or 2 and show less desensitization in cells lacking  $\beta$ -arr1 (Qiu et al., 2007), the enhanced efficacy of  $\delta$ OR agonists in  $\beta$ -arr1<sup>-/-</sup> neurons could be a result of attenuated desensitization. This was assessed *in vitro* by both rapid desensitization in which an agonist is continually perfused while  $\delta$ OR-VDCC inhibition is monitored, and acute desensitization in which the cells are pre-incubated with SNC80, washed, and  $\delta$ OR-VDCC inhibition assessed. Both measures showed a genotype effect (Fig. 2A, B). Firstly, rapid desensitization occurred in both  $\beta$ -arr1<sup>-/-</sup> and <sup>+/+</sup> neurons, but this was less in  $\beta$ -arr1<sup>-/-</sup> neurons ( $p < 0.01$  vs  $\beta$ -arr1<sup>+/+</sup>, Fig. 2A). Secondly, acute desensitization, involving pre-incubation with 50 or 500nM of SNC80 for 10, 30, or 60min, followed by a 1 $\mu$ M SNC80 test dose, showed delayed desensitization after 10min of 50nM SNC80 in  $\beta$ arr1<sup>-/-</sup> neurons but equivalent desensitization thereafter, and no difference in the amount or rate of desensitization induced by 500nM SNC80 (Fig. 2B). In summary, we have found that  $\delta$ OR desensitization was delayed in  $\beta$ -arr1<sup>-/-</sup> neurons. However, when compared with  $\delta$ OR-VDCC inhibition which reached a maximum within 20 – 40s of agonist exposure, the attenuated desensitization, seen by 120s of continual agonist perfusion, cannot explain the enhanced  $\delta$ OR-VDCC inhibition seen in  $\beta$ -arr1<sup>-/-</sup> neurons.

**$\beta$ -arr1 regulation of  $\delta$ OR internalization**—As the arrestins are known to initiate internalization, deleting  $\beta$ -arr1 could delay  $\delta$ OR internalization to increase cell surface receptor number and enhance  $\delta$ OR function. Internalization was examined by flow cytometry using an Allophycocyanin-labeled antibody to the N-terminus of the  $\delta$ OR, the specificity of which was confirmed in cells lacking  $\delta$ ORs (SFig 2). The non-fluorescent parameters of size and granularity were then used to gate on medium-large sized neurons (Fig 2Ci, Walwyn et al., 2004). These cells showed no effect of genotype on basal cell surface receptor levels ( $\beta$ -arr1<sup>-/-</sup>: 89.9 $\pm$ 4.0 of  $\beta$ -arr1<sup>+/+</sup> levels). However, after 10 min of 50 and 500nM, but not 1 $\mu$ M, SNC80, cell surface receptor levels were increased in  $\beta$ -arr1<sup>-/-</sup> ( $p < 0.05$  vs untreated  $\beta$ -arr1<sup>-/-</sup>) but not <sup>+/+</sup> neurons ( $p < 0.05$  vs  $\beta$ -arr1<sup>+/+</sup>, Fig. 2Cii). After 1h of SNC80 (1 $\mu$ M), virally expressed cerulean-labeled  $\delta$ ORs expressed in  $\beta$ -arr1<sup>+/+</sup> and <sup>-/-</sup> neurons, demonstrate significant internalization (SFig 3). As  $\delta$ ORs are dynamically trafficked to and from the cell membrane, the initial increase in  $\delta$ ORs on the cell membrane could reflect delayed internalization and/or enhanced externalization in  $\beta$ -arr1<sup>-/-</sup> neurons (Cahill et al., 2007; Walwyn et al., 2009).

**Accumulation of  $\delta$ ORs on the cell membrane does not account for the enhanced  $\delta$ OR function in  $\beta$ -arr1<sup>-/-</sup> neurons**—Attenuated internalization or enhanced externalization would lead to an accumulation of receptors on the cell membrane, so explaining the increase in  $\delta$ OR-VDCC coupling in  $\beta$ -arr1<sup>-/-</sup> neurons. Lentiviral expression of cerulean-tagged (CFP)  $\delta$ ORs was therefore used to over-express  $\delta$ ORs in  $\beta$ -arr1<sup>-/-</sup> and <sup>+/+</sup> neurons. This did increase  $\delta$ OR-VDCC inhibition, assessed by either DPDPE or SNC80, 1 $\mu$ M ea ( $p < 0.05$  vs endogenous levels, Fig. 2D). However, the increase occurred equally in  $\beta$ -arr1<sup>+/+</sup> and <sup>-/-</sup> neurons suggesting that  $\delta$ OR-VDCC inhibition is sensitive to altered receptor number but that the regulatory role of  $\beta$ -arr1 remains even when these receptors are over-expressed ( $p < 0.05$  vs  $\beta$ -arr1<sup>+/+</sup>).

**$\beta$ -arr1 control of protein export explains the enhanced  $\delta$ OR-VDCC inhibition**—As flow cytometric analysis showed a greater number of  $\delta$ ORs on the cell membrane, it is possible that  $\beta$ -arr1 could control  $\delta$ OR release to the cell membrane in an agonist-dependent manner. Cells were pre-incubated with Brefeldin A, an inhibitor of protein export. This

reduced  $\delta$ OR-VDCC inhibition in  $\beta$ -arr1 $^{-/-}$  neurons to  $+/+$  levels with no effect in  $+/+$  neurons at this concentration ( $p < 0.05$  vs untreated  $\beta$ -arr1 $^{-/-}$ , Fig. 3i). A more specific inhibitor of protein export from the Golgi, Exo1 (0.8 $\mu$ g/ml), reduced  $\delta$ OR-VDCC inhibition in  $\beta$ -arr1 $^{-/-}$  neurons and, when used at a higher concentration (3.2 $\mu$ g/ml), reduced  $\delta$ OR-VDCC inhibition in both  $\beta$ -arr1 $+/+$  and  $^{-/-}$  neurons ( $p < 0.05$  vs untreated, Fig. 3ii). To probe for non-specific effects, GABA $_B$ -VDCC was examined in the presence of Exo1 but no effect found (Fig. 3iii). As  $\delta$ OR-VDCC inhibition is closely correlated with cell surface receptor levels (Walwyn et al., 2005), these data could be interpreted as an Exo1-mediated inhibition  $\delta$ ORs released to the cell membrane in an agonist-dependent manner.

**$\delta$ OR activation of cofilin is  $\beta$ -arr1 dependent**—Both LIMK and cofilin are required for the export of specific proteins from the Golgi to the cell membrane (Salvarezza et al., 2009; von Blume et al., 2009).  $\delta$ ORs could activate cofilin to regulate receptor export via this pathway, and subsequently receptor function.  $\delta$ OR activation of the phosphorylated, inactivated form of cofilin was assessed in mouse embryonic fibroblasts (MEFs). MEFs were chosen for this assay as they express or lack the necessary proteins of this pathway;  $\beta$ -arr1, ROCK, LIMK, and cofilin and have been used to study arrestin-GPCR signaling (Zoudilova et al., 2010) This pathway is also conserved in DRGs (Ahmed et al., 2011). In wildtype MEFs, we found that SNC80 (1 $\mu$ M) reduced the amount of phosphorylated cofilin in  $\beta$ -arr1 $^{-/-}$  (2–30 min;  $p < 0.01$  or 0.01 vs untreated  $\beta$ -arr1 $+/+$ ) but not  $+/+$  MEFs. Together these data show that SNC80 activates cofilin in  $\beta$ -arr1-dependent manner (Fig. 4A).

**ROCK, LIMK and  $\beta$ -arr1 regulation of  $\delta$ OR-VDCC coupling**—As  $\beta$ -arr1 is known to bind with and regulate LIMK (Zoudilova et al., 2007), and LIMK has been shown to control the export of specific proteins from the Golgi (Salvarezza et al., 2009), it is possible that  $\delta$ OR export and therefore function may be regulated by  $\beta$ -arr1. We examined this possibility by inhibiting ROCK, the kinase responsible for phosphorylating and activating LIMK, in neurons containing or lacking  $\beta$ -arr1. The ROCK inhibitor, Y27632, decreased  $\delta$ OR-VDCC inhibition in both  $\beta$ -arr1 $^{-/-}$  and  $+/+$  neurons ( $p < 0.01$  vs untreated  $\beta$ -arr1 $+/+$ ;  $p < 0.001$  vs untreated  $\beta$ -arr1 $^{-/-}$ , Fig. 4B) but had no effect on GABA $_B$ -VDCC coupling (Untreated:  $\beta$ -arr1 $+/+$ : 49.8 $\pm$ 5.3 %,  $\beta$ -arr1 $^{-/-}$ : 46.6 $\pm$ 3.4 %, Y27632 (400ng/ml);  $\beta$ -arr1 $+/+$ : 49.5 $\pm$ 2.2 %,  $\beta$ -arr1 $^{-/-}$ : 51.1 $\pm$ 2.5 %). We then used an approach designed to enhance LIMK activity by including excess LIMK (1 and 2 $\mu$ g/ml) within the intracellular recording solution. The 2 $\mu$ g/ml dose increased  $\delta$ OR-VDCC coupling in both  $\beta$ -arr1 $+/+$  and  $^{-/-}$  neurons ( $p < 0.05$  vs untreated  $\beta$ -arr1 $+/+$  or  $^{-/-}$ , Fig. 4B) demonstrating that LIMK may influence  $\delta$ OR-VDCC inhibition. As LIMK phosphorylation of cofilin can be inhibited by the Serine 3 LIMK peptide (Aizawa et al., 2001), we included this peptide in the intracellular recording solution (S3; 1.6 and 6.4 $\mu$ g/ml). This reduced  $\delta$ OR-VDCC inhibition in  $\beta$ -arr1 $^{-/-}$  neurons ( $p < 0.001$  vs untreated  $\beta$ -arr1 $^{-/-}$ ) but not  $+/+$  neurons (Fig. 4B). Together these data suggest that  $\delta$ OR-VDCC inhibition may be regulated by ROCK, LIMK and  $\beta$ -arr1.

**Actin polymerization influences  $\delta$ OR-VDCC inhibition**—LIMK has been shown to phosphorylate and inhibit cofilin to stabilize the actin cytoskeleton in dendritic spines (Shi et al., 2009). It is possible that LIMK,  $\beta$ -arr1 and cofilin could similarly regulate the stability of the actin ‘tracks’ required for the export of  $\delta$ ORs to the cell membrane. The ability of actin polymerization to affect  $\delta$ OR-VDCC inhibition was therefore examined by manipulating actin polymerization minutes before assessing  $\delta$ OR-VDCC inhibition. Firstly we stabilized the actin cytoskeleton by including Jasplakinolide and Thymosin  $\beta$ 4 within the intracellular recording solution (Bubb et al., 1994; Huff et al., 2001). This increased  $\delta$ OR-VDCC inhibition in  $+/+$  neurons to  $^{-/-}$  levels but had no effect in  $\beta$ -arr1 $^{-/-}$  neurons ( $p < 0.05$  vs untreated  $\beta$ -arr1 $+/+$ , Fig. 4C). Preventing polymerization by including Latrunculin B (LatB, 200ng/ml)(Morton et al., 2000) within the intracellular recording solution reduced the

enhanced  $\delta$ OR-VDCC inhibition in  $\beta$ arr1<sup>-/-</sup> neurons to +/+ levels without affecting GABA<sub>B</sub>-VDCC inhibition ( $p < 0.05$  vs untreated  $\beta$ -arr1<sup>-/-</sup>, Fig. 4C). Together these data demonstrate that manipulations designed to influence actin polymerization can regulate SNC80-VDCC inhibition.

**ORL1-VDCC inhibition is similarly controlled by  $\beta$ -arr1, ROCK, LIMK and actin polymerization**—The previous experiments used  $\delta$ ORs as a model GPCR to examine each component of this regulatory pathway. As ORL1-VDCC inhibition was upregulated in  $\beta$ -arr1<sup>-/-</sup> neurons we next examined whether, similar to  $\delta$ ORs, ORL1-VDCC may be controlled by this ROCK-LIMK pathway in a  $\beta$ -arr1 dependent manner. We assessed the effect of the following inhibitors of this pathway, Y27632 (400ng/ml), S3 (6.4 $\mu$ g/ml), or LatB (200nM), on ORL1-VDCC coupling in  $\beta$ -arr1<sup>-/-</sup> and +/+ DRG neurons. These three compounds reversed the enhanced ORL1-VDCC inhibition in <sup>-/-</sup> neurons ( $p < 0.001$  vs untreated  $\beta$ -arr1<sup>-/-</sup>), but had no effect in +/+ neurons (Fig 4D). These data suggest that ORL1-VDCC coupling may be controlled by ROCK, LIMK, and actin polymerization in the absence of  $\beta$ -arr1<sup>-/-</sup>.

**Basal F-actin incorporation appears unchanged**—As deleting  $\beta$ -arr1 could alter basal LIMK and cofilin activity, this could affect actin turnover throughout the cell. This was assessed by the incorporation of Atto-647N Phalloidin (30nM, 10min) in living DRG neurons that were then fixed and analyzed by Stimulated Emission Depletion (STED) microscopy. No effect of genotype on Phalloidin-incorporation was observed in either the neuronal cell bodies or processes of  $\beta$ -arr1<sup>-/-</sup> vs +/+ neurons suggesting that basal actin polymerization was not altered in  $\beta$ -arr1 DRGs (sFig. 4).

### Behavioral assays provide functional evidence for the role of ROCK and $\beta$ -arr1 in regulating $\delta$ OR and ORL1 function

**$\delta$ OR**—Two well-known behavioral assays of  $\delta$ OR function, locomotion and analgesia, were used to assess the function of this pathway. In the open-field test of locomotion, SNC80 (3mg/kg s.c.) increased the locomotor activity of  $\beta$ -arr1<sup>+/+</sup> mice 20min after the injection. This was reversed by both the ROCK inhibitor (Y27632; 1mg/kg i.p.) and the delta antagonist, NTI (2mg/kg s.c.,  $p < 0.001$  locomotion x treatment, Fig 5Ai). In contrast,  $\beta$ -arr1<sup>-/-</sup> mice showed an enhanced locomotor effect of SNC80 seen both as a more rapid onset, occurring within 10min of the SNC80 injection, and lasting considerably longer than in wild type littermates ( $p < 0.001$  vs  $\beta$ -arr1<sup>+/+</sup>). Similar to +/+ mice, this was reversed by Y27632 and NTI ( $p < 0.001$  locomotion x treatment, Fig. 5Aii). To assess whether this inhibitory profile of Y27632 was due to non-specific suppression of locomotor behavior, we tested the effect on Y27632 on the locomotor effects of fentanyl, a specific mu opioid receptor agonist. Fentanyl (0.4mg/kg s.c.) induced an equal hyperlocomotor response in both genotypes which was not altered by Y27632 (1mg/kg i.p., sFig 5A). No gender based difference in locomotion were observed in either the SNC80 ( $p = 0.69$  locomotion x gender) or fentanyl-treated groups ( $p = 0.54$  locomotion x gender).

In the second behavioral test of  $\delta$ OR function, the analgesic effect of SNC80 was assessed by mechanical pain in which the number of paw withdrawals from 10 applications of a 2g von Frey filament was measured. We found that basal thresholds were not influenced by genotype, and that wildtype mice showed a brief analgesic effect of SNC80 at the 20 ( $p < 0.05$  response x treatment), but not 40min timepoint (5mg/kg s.c., Fig. 5Bi). In comparison,  $\beta$ -arr1<sup>-/-</sup> mice showed enhanced analgesia, or fewer responses 20 and 40min after SNC80 ( $p < 0.001$  response x treatment). This effect of SNC80 was reversed by Y27632 (1.5mg/kg) and NTI (4mg/kg, Fig 5Bii).

SNC80 produced some analgesia in naïve wildtype mice (Fig 5bi) but, as chronic inflammatory pain results in enhanced  $\delta$ OR analgesia (Pradhan et al., 2013), we next examined the role of ROCK in modulating  $\delta$ OR function in inflammatory pain. We injected saline or Y27632 (10mg/kg i.p.) prior to injecting Complete Freund's Adjuvant (CFA, 10 $\mu$ l) in the left hindpaw of wildtype mice, and at 12h intervals thereafter. After 48h we assessed the effect of SNC80 (1 and 5mg/kg s.c.) on the mechanical pain threshold in Y27632 and saline-treated groups. We found that Y27632 reduced the ability of SNC80 to relieve the mechanical hyperalgesia associated with chronic inflammatory pain ( $p < 0.05$  vs vehicle, Fig. 5C), highlighting the involvement of ROCK in the functional upregulation of  $\delta$ ORs seen in this model.

We also assessed whether genetic background influenced the  $\beta$ -arr1 phenotype. Mice backcrossed to the C57Bl/6 background and lacking  $\beta$ -arr1 showed a similar enhanced response to SNC80 in both locomotor and acute pain studies (sFig 5B, C). Furthermore this effect of SNC80 in  $\beta$ -arr1 $^{-/-}$  mice was specific to  $\beta$ -arr1; SNC80 induced locomotion was similar in  $\beta$ -arr2 $^{-/-}$ ,  $\beta$ -arr2 $^{+/+}$  or  $\beta$ -arr1 $^{+/+}$  mice (sFig 5B).

**ORL1**—We assessed the role of this pathway in modulating ORL1 function by measuring the locomotor effect of the ORL1 agonist, Ro 65-6570. This agonist is similar to Ro 64-6198, which has been shown to either induce hypolocomotion or have no locomotor effect in wildtype mice (Shoblock, 2007). Although wildtype mice showed no effect of Ro 65-6570 (1.5mg/kg i.p.),  $\beta$ -arr1 $^{-/-}$  mice showed a hypolocomotor response ( $p < 0.05$  locomotion  $\times$  treatment) which was reversed by both Y27632 (0.5mg/kg) and the ORL1 antagonist, J-113397 ( $p < 0.05$  vs Ro 65-6570, Fig 6). Together these data show enhanced ORL1 function in  $\beta$ -arr1 $^{-/-}$  mice which can be reversed by inhibiting ROCK.

## Discussion

In being able to scaffold with cofilin, the inactivating kinase, LIMK, and activating phosphatase, slingshot (SSL) or chronophin, arrestins can control the activity of cofilin and therefore actin polymerization (Condeelis, 2001; Xiao et al., 2010; Zoudilova et al., 2007; Zoudilova et al., 2010). This pathway enables a dynamic remodeling of the actin cytoskeleton in response to a chemotactic stimulus (Nishita et al., 2005) and, as we show here, modulates the function of some GPCRs;  $\delta$ OR and ORL1.

### A model of the pathway

Based on GPCR inhibition of Ca<sup>2+</sup> channels, a voltage-dependent and G $\beta\gamma$ -mediated response that modulates neurotransmitter release, we propose the following model (Fig. 7). Under normal or wildtype conditions, agonist binding to an agonist such as the  $\delta$ OR, activates cofilin through the RhoA-ROCK-LIMK pathway. However, as  $\beta$ -arr1 is associated with LIMK and one of the cofilin phosphatases such as SSL (Xiao et al., 2010; Zoudilova et al., 2010), an enhanced but local activation of cofilin occurs. We propose that this results in controlled severing of F-actin and measured actin turnover to regulate the stability of a subset of actin filaments within the Golgi. This in turn, controls the rate of export of  $\delta$ OR-containing cargo vesicles from the Golgi to the cell membrane to allow a limited response to a  $\delta$ OR agonist such as SNC80 (Fig. 7A). In the absence of  $\beta$ -arr1, SNC80 similarly activates LIMK through the Rho-ROCK pathway but, without the regulatory control of  $\beta$ -arr1, cofilin is not dephosphorylated and activated by SNC80 (Lin et al., 2003). We propose that this leaves stable actin 'tracks' in place resulting in enhanced export of  $\delta$ ORs from the Golgi to the plasma membrane to enhance function (Fig 7B). This pathway can be blocked by inhibiting ROCK, the kinase responsible for phosphorylating LIMK and inactivating cofilin. In preventing agonist-induced activation of this pathway, we propose that agonist-induced

turnover of a subset of actin filaments within the Golgi does not occur and additional receptors are not released to the cell membrane. Without this activation, the functional effect of the agonist is severely limited (Fig. 7C).

### **$\delta$ OR trafficking and function**

$\delta$ OR-VDCC inhibition is a rapid, voltage-dependent cascade that can be completely reversed by a high-voltage pre-pulse (data not shown). We have previously found that  $\delta$ OR-VDCC inhibition is positively correlated with  $\delta$ OR cell surface receptor levels (Walwyn et al., 2005). Furthermore increased  $\delta$ OR-VDCC coupling is associated with enhanced  $\delta$ OR function (Pradhan et al., 2013) and, conversely, reduced  $\delta$ OR function is associated with reduced cell surface receptor levels (Scherrer et al., 2006). Together this suggests a close relationship between cell surface receptor levels and  $\delta$ OR functionality, assessed either *in vitro* or *in vivo*. The data presented here suggest that the ROCK-LIMK- $\beta$ -arr1 pathway is a critical mediator of this relationship and is required to obtain an initial functional response. Thereafter, the co-operative roles of  $\beta$ -arr1 in both desensitizing and modulating  $\delta$ OR trafficking, may further regulate  $\delta$ OR signaling.

### **ORL1 function**

Similar to  $\delta$ ORs, our data suggest that  $\beta$ -arr1 also regulates ORL1 function in a ROCK, LIMK and actin-dependent manner. However, this regulation is not evident in wildtype mice. This could be that ROCK, LIMK and  $\beta$ -arr1 inhibits ORL1 function in wildtype neurons and mice to non-detectable levels. This is suggested from the low levels of ORL1-VDCC coupling in wildtype neurons (Fig 1) and the lack of a locomotor response to the ORL1 agonist used (Fig 6). Interestingly ORL1 function has often been described in association with a pharmacological or behavioral stimulus (Bebawy et al., 2010; Shoblock, 2007) suggesting that this receptor, similar to  $\delta$ ORs, may be upregulated under certain conditions.

### **Other receptors or channels that may be regulated by $\beta$ -arr1, cofilin and LIMK**

This fine-tuned control of actin polymerization to affect  $\delta$ OR and ORL1 function may similarly control ligand-dependent activation of other GPCRs such as dopamine receptor 1, neurotensin or PAR<sub>2</sub> which are recruited from intracellular stores to the cell membrane 'upon demand' (Brismar et al., 1998; Hein et al., 1994; Perron et al., 2006). Interestingly many of these receptors share a common post-endocytic fate of being degraded once internalized suggesting that regulating the rate of receptor release may be important to allow sufficient GPCR activation. In providing a ligand-dependent spatio-temporal regulation of actin filaments,  $\beta$ -arr1 may also regulate the function of ligand-gated ion channels which are may be rapidly trafficked to and from the synapse upon demand.

### **Other regulators of the pathway**

We have examined GPCR activation of this pathway but it is also possible that other receptors or molecules may be involved. For example, bradykinin or arachidonic acid has been shown to enhance  $\delta$ OR function (Patwardhan et al., 2005; Pettinger et al., 2013; Rowan et al., 2009). As kinins are involved in the hyperalgesic response to chronic pain (Ferreira et al., 2002), it is possible that bradykinin may activate this pathway in the CFA model of chronic pain.

### **Conclusion**

Following agonist-receptor activation of downstream signaling cascades, GPCRs typically desensitize, becoming unable to induce the same signaling profile. In contrast, we have found that agonist binding with select GPCRs results in a functional upregulation of the

receptor. This increases GPCR-Ca<sup>2+</sup> channel inhibition and agonist-mediated behaviors demonstrating how this novel regulatory pathway allows these receptors to maintain or enhance receptor function.

## Methods

### Animals

All animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and followed institutionally approved animal care and use protocols. Mice lacking the  $\beta$ -arr1 (Conner et al., 1997) or  $\delta$ OR (Filliol et al., 2000) and were fully back-crossed into the 129S6/SvEv or C57Bl/6 background. All animals used for the cellular experiments were bred from homozygous matings within 1 generation of heterozygous matings. For the behavioral experiments, 2–3 month equally distributed males and females were generated from heterozygous matings. Unless noted otherwise, all experiments used the 129S6/SvEv  $\beta$ arr1 line.

### Compounds

Unless noted otherwise, all chemicals were obtained from Sigma; St Louis, MO, except for Y27632; Selleckchem Houston, TX, SNC80, Naltrindole, ICI 174–864, Nociceptin, J113397, JTC801; R&D, Minneapolis, MN, S3; Anaspec, Fremont, CA, and LIMK; Cytoskeleton, Denver, CO.

### Dorsal root ganglion neuron cultures

DRGs were harvested from early postnatal (p0–1) mice or adult (6–8 wk old) mice. The DRGs were enzymatically and physically dissociated and plated in different formats and densities for different experiments. For electrophysiology experiments  $5 \times 10^4$  dissociated cells were plated on poly-D-lysine (Sigma, MO) and laminin- (Becton Dickinson, Bedford, MA) coated coverslips (10 mm diameter) in the center of 35mm MatTek dishes (Ashland, MA). Lentiviral transduction was performed at the time of plating by adding 50ng p24/plate to each plate. For flow cytometry experiments  $1 \times 10^6$  cells were plated in a similarly-coated 15mm MatTek dish. The cells were cultured in 2ml of Neurobasal A, B27, Glutamax (0.5mM) and antibiotic-antimycotic (12U/ml,) media (Life Technologies, Carlsbad, CA) containing NGF (10 $\mu$ g/ml, Roche, Indianapolis, IN) and kept at 37°C and 5% CO<sub>2</sub>. Electrophysiological assessment of endogenous  $\delta$ OR function was performed within 24h of plating, and those in which receptors were over-expressed, 72–96h thereafter. Flow cytometry was performed within 48h of plating.

### Electrophysiology

The whole-cell patch-clamp technique was used to record voltage-dependent Ca<sup>2+</sup> channel (VDCC) activity from DRG neurons (Axopatch 200A amplifier, Molecular Dynamics, CA) as previously described (Walwyn et al, 2007). At the time of recording the culture medium was replaced by an external solution that contained (in mM): 130 TEA-Cl, 10 CaCl<sub>2</sub>, 5 HEPES, 25 D-glucose and  $2.5 \times 10^{-4}$  tetrodotoxin at pH 7.2. Recording electrodes contained (in mM): 105 CsCl, 40 HEPES, 5 D-glucose, 2.5 MgCl<sub>2</sub>, 10 EGTA, 2 Mg<sup>2+</sup>-ATP and 0.5 Na<sup>+</sup>-GTP, pH 7.2. Ca<sup>2+</sup> currents were activated by depolarizing neurons from –80mV to 10mV for 100ms at 20s intervals. Recordings that exhibited marked rundown were discarded. Stable recordings were fitted by a linear function to compare, by extrapolation, control current amplitude to the current amplitude recorded in the presence of the agonist. Data are expressed as mean $\pm$ SEM and were compared using ANOVA with a posthoc Scheffe test.



### Cofilin dephosphorylation

Wild type (wt) mouse embryonic fibroblasts (MEFs) or  $\beta$ arr1<sup>-/-</sup> MEFs were treated with SNC80 for 0–120 min, lysed and analyzed by SDS-PAGE followed by western blotting with antibodies to phospho-cofilin (rabbit polyclonal, Cell Signaling, 1:1000) or total cofilin (mouse monoclonal, BD Biosciences, 1:500) and analyzed as previously described (Zoudilova et al., 2007).

**Statistical Analysis**—The data are expressed as the mean±SEM and differences between groups determined by one-way ANOVA (Prism v5.03) with significance accepted at  $p<0.05$ .

### Flow cytometry

After 48h *in vitro*  $\beta$ arr1<sup>+/+</sup> and <sup>-/-</sup> or  $\delta$ OR<sup>+/+</sup> and <sup>-/-</sup> DRG neurons were harvested and processed for  $\delta$ OR fluorescent labeling in non-fixed cells using a primary antibody to the N-terminus of the  $\delta$ OR (MBL, Woburn, MA) and an Allophycocyanin (APC)-conjugated secondary antibody (BD Biosciences, San Jose, CA) as previously described (Walwyn et al., 2009).

**Statistical Analysis**—The data are expressed as the mean±SEM and differences between groups determined by one-way ANOVA or paired student's T-test with significance accepted at  $p<0.05$  (Analyse-it Software Ltd., United Kingdom).

### Behavioral tests

Two-three month old  $\beta$ -arr1<sup>-/-</sup> and <sup>+/+</sup> littermates of both genders were used for the following behavioral effects.

**a. Locomotion**—As SNC80 is known to increase locomotor behavior, this effect was measured after 30min of habituation to the test environment and the effect of SNC80, following the injection of the  $\delta$ OR antagonist, naltrindole, the ROCK inhibitor Y27632 or vehicle, delivered subcutaneously (s.c.) or intraperitoneally (i.p.), measured over the following 90min. This behavior was recorded in 27×27 cm plastic boxes by live tracking using an infra-red camera and Ethovision XT (Noldus, Leesburg, VA). After 30min of habituation the mice were injected with the first of 2 injections, the second followed 15min later, and locomotion was measured for the following 90min. As ORL1 agonists can induce a hypolocomotor response, this effect was tested by injecting a bio-available ORL1 agonist, Ro 65-6570 (1.5 mg/kg i.p.) after 15 min of habituation and for the following 45min. The ROCK inhibitor Y27632, the ORL1 antagonist J-113397, or vehicle, were injected 15min prior to Ro 65-6570.

**b. Mechanical pain**—After 2 days of habituation to the test environment, the number of responses, defined as a lifting or shaking of the paw upon stimulation of the plantar surface, out of a total of 10 repetitions, to the 2g von Frey filament was measured. Basal responses to the 2g filament were acquired prior to the injections and the effect tested 20 and 40min thereafter.

**CFA**—Wildtype mice were habituated for 3 days prior to obtaining the basal mechanical threshold by the up/down method (Chaplan et al., 1994). Saline or Y27632 (10mg/kg i.p.) was injected prior to assessing the basal response, prior to the CFA injection (10 $\mu$ l in the left hindpaw), and at 12h intervals thereafter. After 48h, the mechanical pain threshold was assessed and the effect of SNC80 (1 and 5mg/kg s.c.) on determined.

**Statistical analysis**—Data are expressed as mean±SEM and differences between groups analyzed by ANOVA with repeated measures and factorial analysis at each timepoint (Stat View v5).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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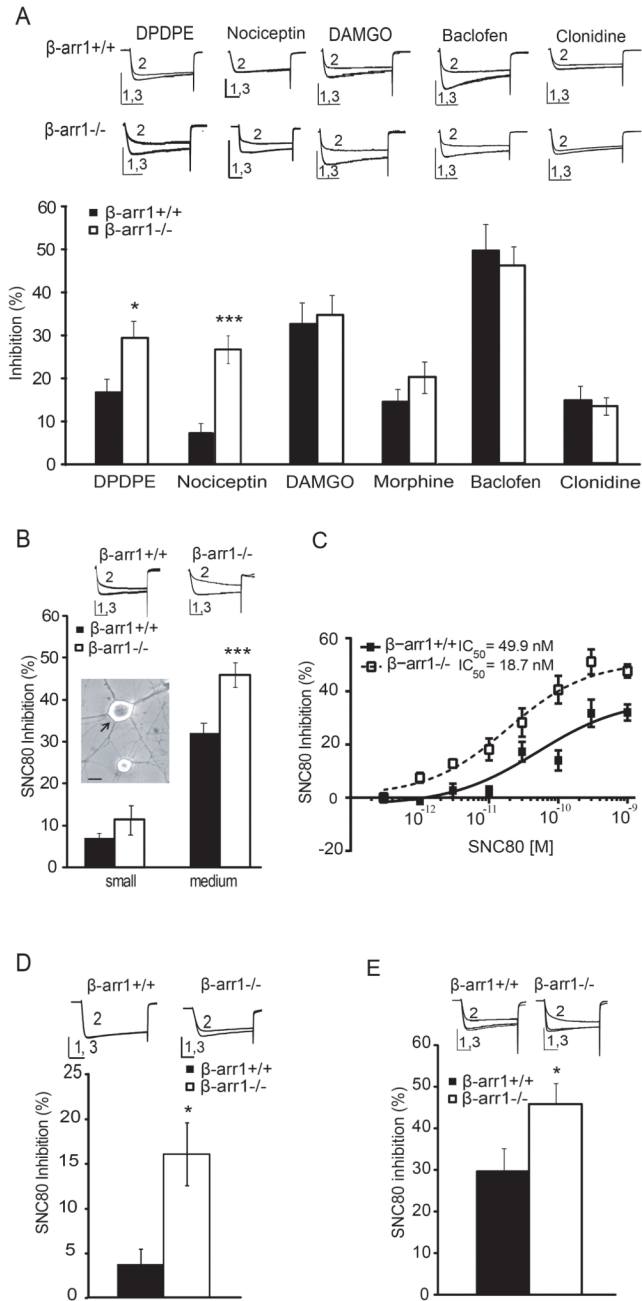
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### Highlights

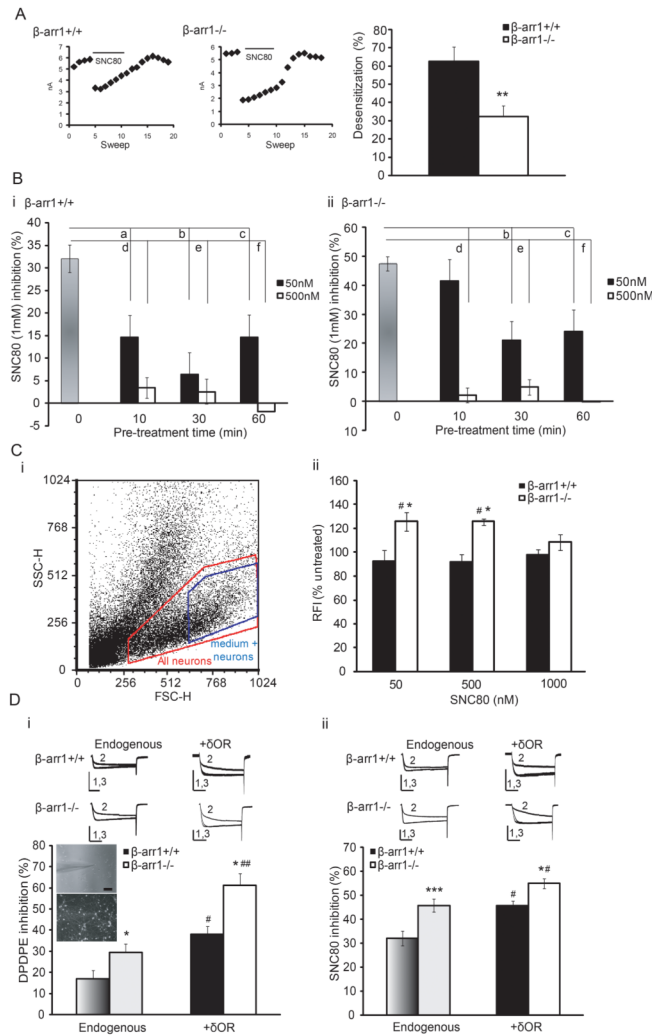
- $\delta$ OR activation of the ROCK-LIMK-cofilin pathway rapidly regulates  $\delta$ OR function
- $\beta$ -arrestin1 deletion increases, while ROCK inhibition suppresses,  $\delta$ OR function
- Activation of this pathway can alter  $\delta$ OR-dependent behaviors
- ORL1 is similarly regulated by this ROCK- $\beta$ -arrestin 1 pathway



**Fig. 1.  $\delta$ OR and ORL1 function is enhanced in dorsal root ganglia neurons lacking  $\beta$ -arr1**

A. The whole cell patch clamp technique was used to examine VDCC inhibition by  $G_{o/i}$  coupled receptors. a. DPDPE, a peptidergic  $\delta$ OR agonist, and nociceptin, an ORL1 agonist, demonstrated enhanced VDCC coupling in  $\beta$ -arr1<sup>-/-</sup> vs  $\beta$ -arr1<sup>+/+</sup> DRG neurons whereas VDCC inhibition by agonists of the mu receptor, DAMGO and morphine (1 $\mu$ M ea), the GABA<sub>B</sub> receptor, Baclofen (50 $\mu$ M), and the  $\alpha_{2A}$  receptor, Clonidine (10 $\mu$ M) was not different across genotypes. B. Further analysis of  $\delta$ OR-VDCC inhibition showed enhanced  $\delta$ OR inhibition of VDCCs by the non-peptidergic  $\delta$ OR agonist, SNC80, in medium (shown by the arrow), but not small, early post-natal medium-sized  $\beta$ -arr1<sup>-/-</sup> vs  $\beta$ -arr1<sup>+/+</sup> DRG neurons in the SvEv129SJ background, scalebar=10 $\mu$ m. C. The concentration response curve demonstrated enhanced efficacy and potency of SNC80 in  $\beta$ -arr1<sup>-/-</sup> vs  $\beta$ -arr1<sup>+/+</sup> neurons ( $p < 0.001$ ). D. Similar

to neurons from mice back-crossed into the SvEv129SJ background, medium-sized DRG neurons from early post-natal  $\beta$ -arr1<sup>-/-</sup> in the C57Bl/6 background showed enhanced SNC80-VDCC inhibition. E. Similar to early post-natal DRG neurons, medium-sized DRG neurons from adult mice showed increased SNC80-VDCC inhibition in  $\beta$ -arr1<sup>-/-</sup> vs  $\beta$ -arr1<sup>+/+</sup> neurons. All data are shown as mean $\pm$ SEM, \* $p$ <0.05 vs  $\beta$ -arr1<sup>+/+</sup>, \*\* $p$ <0.001 vs  $\beta$ -arr1<sup>+/+</sup>, \*\*\* $p$ <0.001 vs  $\beta$ -arr1<sup>+/+</sup>,  $n$ =6–27 per data point. All exemplar currents depict the current before (1), during (2) and after agonist (3) application, and the scale bars show time, 20ms, on the x-axis, and current on the y axis; 5 or 10nA for early post-natal or adult DRG neurons respectively.

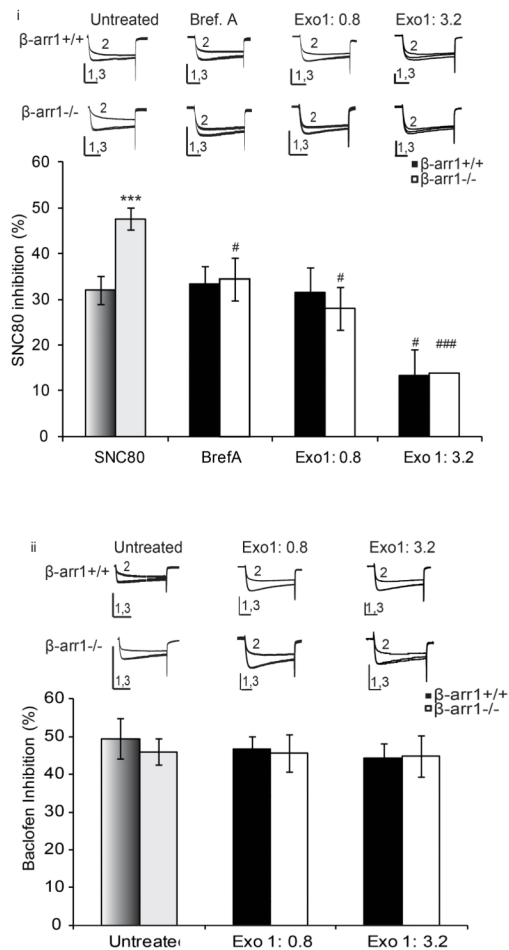


**Fig. 2.  $\beta$ -arr1 regulates  $\delta$ OR desensitization and internalization but this does not explain the enhanced  $\delta$ OR-VDCC coupling in  $\beta$ -arr1<sup>-/-</sup> neurons**

**A.** Rapid desensitization: Once complete inhibition of VDCCs was obtained by SNC80, further perfusion with SNC80 desensitized this inhibition. This is shown by the decline in the peak current amplitude over 120s in the left panels. Although both  $\beta$ -arr1<sup>+/+</sup> and <sup>-/-</sup> neurons desensitized,  $\beta$ -arr1<sup>-/-</sup> neurons showed less desensitization,  $**p < 0.01$ ,  $n = 5-10$ . **B.** Acute desensitization: Whereas pre-incubation of DRGs with 50 and 500nM SNC80 for 10min desensitized  $\delta$ OR-VDCC inhibition at all time points in <sup>+/+</sup> neurons, <sup>-/-</sup> neurons did not desensitize after 10min of 50nM SNC80 but desensitized thereafter and showed equivalent desensitization at all timepoints when pre-incubated in 500 nM SNC80.  $\beta$ -arr1<sup>+/+</sup>; a, b, c:  $p < 0.05$  vs untreated, d, e:  $p < 0.0001$  vs untreated, f:  $p < 0.001$  vs untreated,  $\beta$ -arr1<sup>-/-</sup>; b:  $p < 0.001$  vs untreated, c:  $p < 0.001$  vs untreated, d, e, f:  $p < 0.0001$  vs untreated,  $n = 6-7$  per data point. Columns in shades of grey, Bi; <sup>+/+</sup> or Bii; <sup>-/-</sup>, indicate data shown in Fig 1. **C.** Internalization assessed by flow cytometry in  $\beta$ -arr1<sup>-/-</sup> and <sup>+/+</sup> DRG neurons using an N-terminal antibody and Allophycocyanin (APC)-conjugated secondary antibody. i The  $\delta$ OR-labeled non-fixed samples were initially sorted by size (FSC-H) and granularity (SSC-H) to select the medium-large DRG neurons ( $>25 \mu\text{m}$  in diameter Walwyn et al., 2004). APC-labeled  $\delta$ ORs were then selected after removing non-specific background labeling and all samples of each experiment analyzed by these parameters. ii. Although basal cell surface

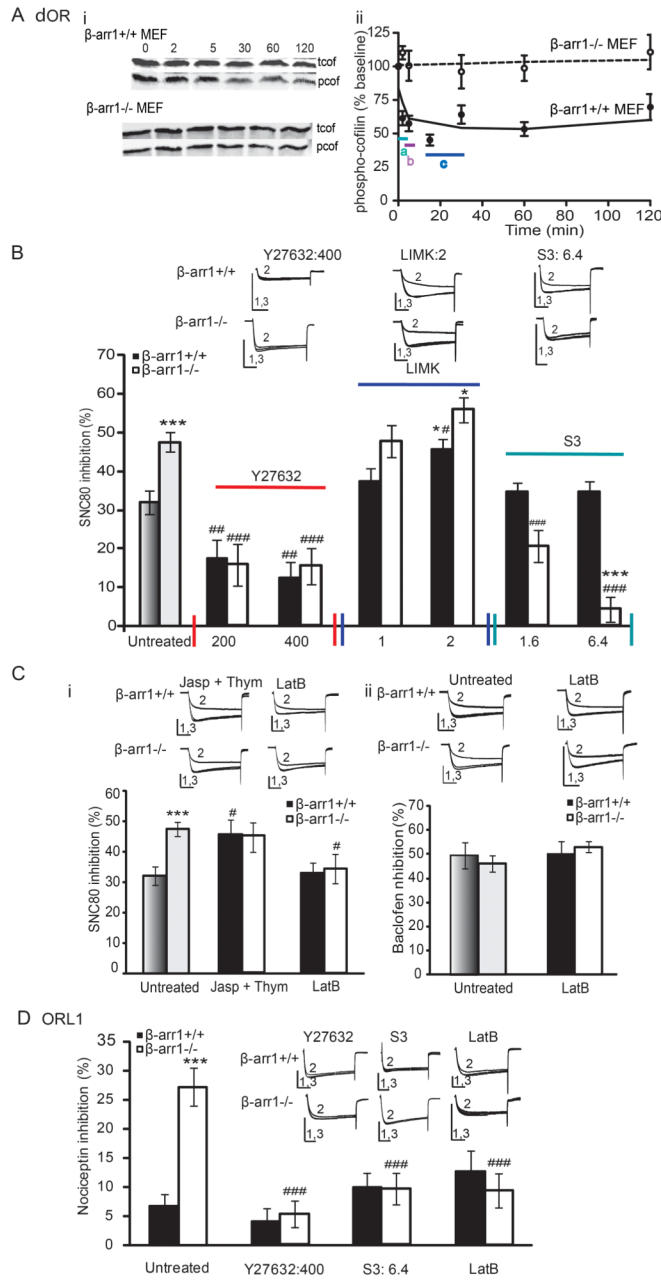


$\delta$ OR levels were unaffected by the  $\beta$ -arr1 $^{-/-}$  deletion, 50 and 500, but not 1000, nM SNC80 increased cell surface  $\delta$ OR levels in  $\beta$ arr1 $^{-/-}$  DRGs. #p<0.05 vs untreated  $\beta$ -arr1 $^{-/-}$ , \*p<0.05 vs  $\beta$ -arr1 $^{+/+}$ , same time point, n=4–8 per data point. D. A slower rate of internalization could enhance  $\delta$ OR-VDCC inhibition by increasing the number of  $\delta$ ORs on the plasma membrane. This was assessed by over-expressing Cerulean (CFP)-labeled  $\delta$ ORs (Walwyn et al., 2009). This increased  $\delta$ OR-VDCC inhibition equally in both  $\beta$ -arr1 $^{+/+}$  and  $^{-/-}$  neurons tested with either DPDPE or SNC80 (1 $\mu$ M ea). \*p<0.05, \*\*p<0.01 and \*\*\*p<0.001 vs  $\beta$ -arr1 $^{+/+}$ , #p<0.05, ## p<0.01 vs endogenous  $\delta$ ORs, n=6–18 per data point. All exemplar currents depict the current before, during and after agonist application (1, 2 and 3) and the scale bars show time, 20ms, and current, 5nA on the x- and y-axes respectively. Columns in dark ( $\beta$ -arr1 $^{+/+}$ ) or light ( $\beta$ -arr1 $^{-/-}$ ) shades of grey indicate data shown in Fig 1. All data are shown as mean $\pm$ SEM.



### Fig. 3. $\beta$ -arr1 control of protein export explains enhanced $\delta$ OR-VDCC inhibition

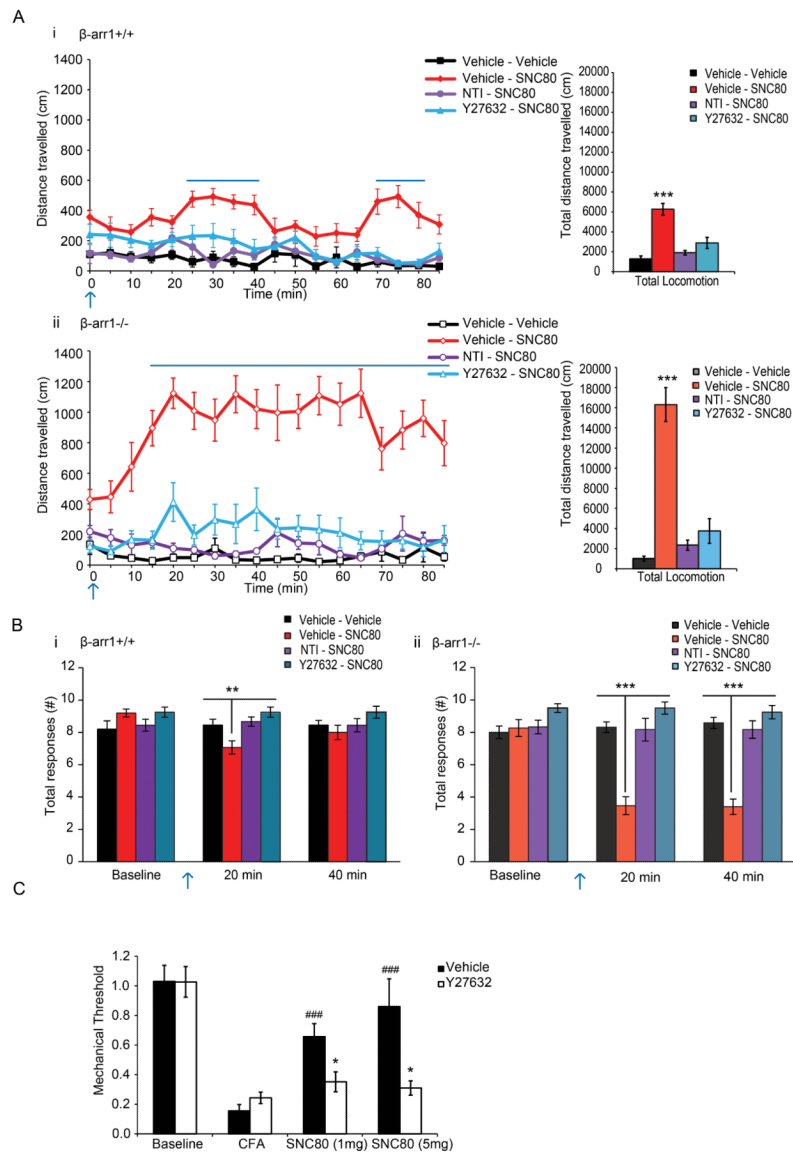
The increase in cell surface receptor number after 50 and 500nM SNC80 (Fig. 2C) could reflect  $\beta$ -arr1 control of  $\delta$ OR release to the plasma membrane. This was assessed by inhibiting protein export i. DRGs were pre-treated with Brefeldin A (Bref. A, 1 $\mu$ g/ml 30min pre-treatment) to inhibit protein release from the ER, and Exo1, a more specific inhibitor of protein release from the Golgi (0.8 and 3.2 $\mu$ g/ml, included in the intracellular recording solution). Both BrefA and Exo1 (0.8 $\mu$ g/ml) reduced the enhanced SNC80 inhibition of VDCCs in  $\beta$ -arr1 $^{-/-}$  to  $+/+$  levels but the higher dose of Exo1 (3.2 $\mu$ g/ml) reduced SNC80-inhibition in both  $^{-/-}$  and  $+/+$  neurons ii. Exo1 did not affect GABA $_B$ -VDCC inhibition, assessed by Baclofen (50  $\mu$ M). All data are shown as mean $\pm$ SEM, # $p$ <0.05 vs untreated  $\beta$ -arr1 $+/+$  or  $^{-/-}$ . \*\*\* $p$ <0.001 vs  $\beta$ -arr1 $+/+$ , n=6–12 per data point All exemplar currents depict the current before, during and after agonist application (1, 2 and 3) and the scale bars show time, 20ms, and current, 5nA on the x- and y-axes respectively. Columns in dark ( $\beta$ -arr1 $+/+$ ) or light ( $\beta$ -arr1 $^{-/-}$ ) shades of grey indicate data shown in Fig 1.



**Fig. 4.  $\delta$ OR and ORL1 inhibition of VDCCs is regulated by LIMK, ROCK and actin polymerization**

$\delta$ OR: A. The  $\delta$ OR agonist, SNC80, activates cofilin: Wild-type MEFs (wt MEFs) or  $\beta$ -arr1<sup>-/-</sup> MEFs were treated with SNC80 for 0–120min and lysates analyzed by Western blot. ai. Representative western blots of phosphorylated cofilin (pcof) and total cofilin (tcof) in wild type wt MEFs (upper two panels) and  $\beta$ arr1<sup>-/-</sup> MEFs (lower two panels) after SNC80 treatment. aii. SNC80 (1 $\mu$ M) decreased phospho-cofilin after 2, 5, 15 and 30min in wt MEFs (closed circles) whereas  $\beta$ arr1<sup>-/-</sup> MEFs (open circles) showed no change. Values on the y-axis represent phospho-cofilin normalized to basal levels at each time point shown on the x-axis. a; p<0.001, b; p<0.01, c; p<0.01 vs untreated wt MEFs. B i. The ROCK inhibitor, Y27632 (200 and 400ng/ml) reduced  $\delta$ OR-VDCC coupling in  $\beta$ -arr1<sup>-/-</sup> and +/+ neurons. Excess LIMK (2 $\mu$ g/ml) increased SNC80-VDCC inhibition in both  $\beta$ -arr1 +/+ and -/-

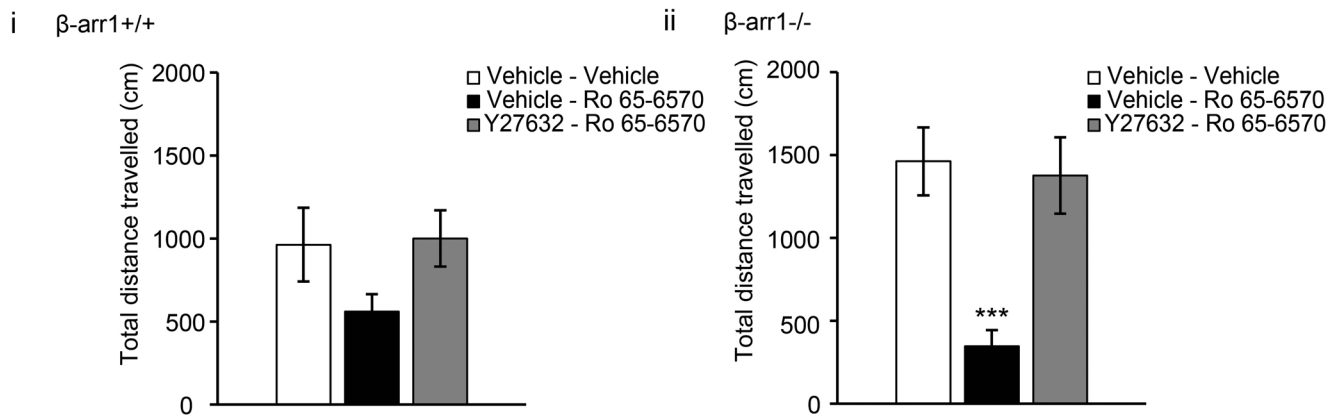
neurons although SNC80-VDCC inhibition remained higher in  $\beta$ -arr1<sup>-/-</sup> neurons. LIMK phosphorylation of cofilin can be prevented by the Serine 3 peptide. This decreased SNC80-VDCC inhibition in  $\beta$ -arr1<sup>-/-</sup> neurons but not <sup>+/+</sup> neurons (S3: 1.6 and 6.4ng/ml). C. i. Jasplakinolde and Thymosin B4 (200ng/ml), enhancers of actin polymerization, increased SNC80-VDCC inhibition in  $\beta$ -arr1<sup>+/+</sup> to <sup>-/-</sup> levels whereas Latrunculin B (LatB: 200ng/ml), an inhibitor of actin polymerization, reversed the increased  $\delta$ OR-VDCC inhibition in  $\beta$ -arr1<sup>-/-</sup> to <sup>+/+</sup> levels. ii. LatB did not alter GABA<sub>B</sub>-VDCC inhibition assessed by Baclofen (50 $\mu$ M). ORL1: D. The increase in ORL1-VDCC inhibition in  $\beta$ -arr1<sup>-/-</sup> DRG neurons was reversed by the ROCK inhibitor, Y27632 (400ng/ml), the LIMK inhibitory peptide, S3 (6.4ng/ml), and Latrunculin B (200ng/ml). No effect of these manipulations was seen in <sup>+/+</sup> neurons. All data are shown as mean $\pm$ SEM, \* $p$ <0.05, \*\*\* $p$ <0.001 vs  $\beta$ -arr1<sup>+/+</sup>, ##, ### $p$ <0.01, or 0.001 vs untreated, matched genotype,  $n$ =6–12 per data point. All exemplar currents depict the current before, during and after agonist application (1, 2 and 3) and the scale bars show time, 20ms, and current, 5nA on the x- and y-axes respectively. Columns in dark ( $\beta$ -arr1<sup>+/+</sup>) or light ( $\beta$ -arr1<sup>-/-</sup>) shades of grey indicate data shown in Fig 1.



**Fig. 5.  $\delta$ OR behaviors are regulated by  $\beta$ -arr1 and LIMK**

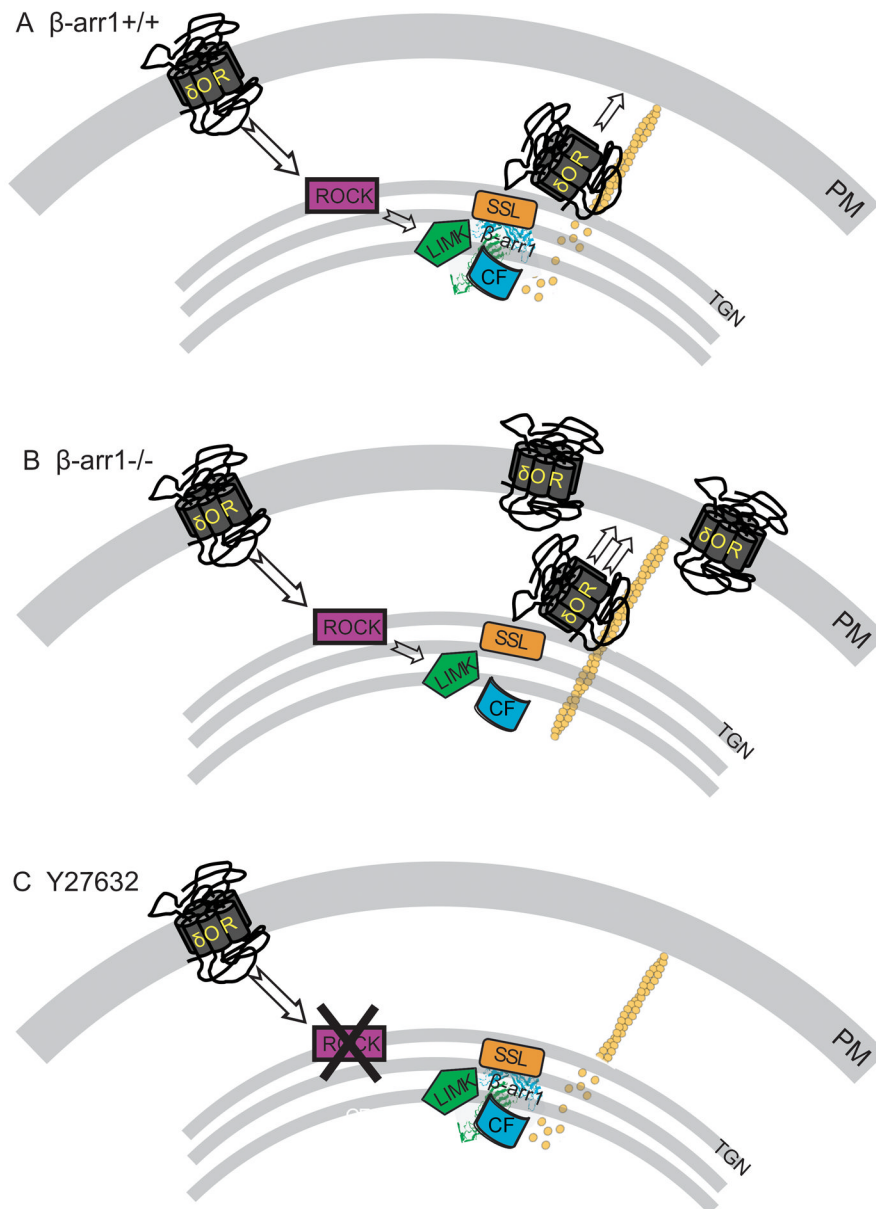
A. Two behavioral assays were used to assess  $\delta$ OR function. i. Locomotion.  $\beta$ -arr1<sup>+/+</sup> mice showed a hyperlocomotor response to subcutaneously administered SNC80 (3 mg/kg s.c.,  $p < 0.001$ ,  $F_{5,28} = 13.125$ , locomotion x treatment), that was reversed by both the ROCK inhibitor (Y27632 1mg/kg i.p.) and the delta antagonist, naltrindole (2mg/kg s.c.).  $\beta$ -arr1<sup>-/-</sup> mice also showed a hyperlocomotor effect of SNC80 ( $p < 0.001$ ,  $F_{5,26} = 31.19$  locomotion x interaction), but this was enhanced ( $p < 0.001$ ,  $F_{1,9} = 25.79$  vs.  $\beta$ -arr1<sup>+/+</sup>), occurred more rapidly than the wildtype mice ( $p < 0.001$ ,  $F_{17,153} = 4.051$  vs.  $\beta$ -arr1<sup>+/+</sup>) and lasted for the duration of the test, 90min. This was similarly reversed by both Y27632 and naltrindole. The data show time (min) on the x-axis and distance traveled (cm) on the y-axis. The blue arrow represents the second of the two injections. The bar graphs depict the total locomotion over time. B. Mechanical analgesia was measured by the number of responses, out of a total of 10 applications of the same von Frey filament (2g), to the plantar surface of the right hind paw.  $\beta$ -arr1<sup>+/+</sup> mice showed a small effect of SNC80 (5mg/kg s.c.,  $p < 0.05$ ,  $F_{3,39} = 3.388$ , response x treatment) at the 20min ( $p < 0.01$ ,  $F_{3,39} = 6.525$ ), but not the 40min timepoint.  $\beta$ -arr1<sup>-/-</sup>

mice, on the other hand, showed a greater response at both the 20 and 40min timepoints after SNC80 administration ( $p < 0.001$ ,  $F_{2,37} = 43.37$ , response x treatment). Y27632 ( $\beta$ -arr1+/+:  $p < 0.01$  vs. SNC80,  $\beta$ -arr1-/-:  $p < 0.001$  vs. SNC80) and naltrindole ( $\beta$ -arr1+/+:  $p < 0.01$  vs. SNC80,  $\beta$ -arr1-/-:  $p < 0.001$  vs. SNC80) reversed the antinociceptive effect of SNC80 in both  $\beta$ -arr1+/+ and  $\beta$ -arr1-/- mice. C. We have previously shown that Complete Freund's Adjuvant (CFA) induced chronic pain decreases the mechanical threshold in the von frey test of allodynia ( $p < 0.001$ ,  $F_{1,25} = 114.17$  vs. baseline) in adult C57Bl6/J mice. This decrease is associated with a functional upregulation of  $\delta$ ORs (Pradhan et al., 2013) as it can be reversed by SNC80 (1 or 5 mg/kg s.c.;  $p < 0.001$ ,  $F_{1,23} = 33.84$  threshold x treatment) in wildtype mice. Administration of Y27632 (10 mg/kg i.p.) prior to injecting CFA and at 12h intervals thereafter, did not alter the resultant hyperalgesia ( $p = 0.563$ ,  $F_{1,25} = 0.34$  vs. vehicle), but did inhibit the effect of SNC80 (1 and 5 mg/kg; \* $p < 0.05$  vs. vehicle, ### $p < 0.001$  vs. CFA). All data are shown as mean  $\pm$  SEM.



**Fig. 6. ORL1 behavioral responses are similarly regulated by  $\beta$ -arr1 and LIMK**

The bio-available ORL1 agonist, Ro 65-6570 (1.5mg/kg i.p.) did not alter the locomotor profile of  $\beta$ -arr1<sup>+/+</sup> mice ( $p=0.54$ ,  $F_{3,23}=0.721$  locomotion x treatment) but induced a hypolocomotor response in  $\beta$ -arr1<sup>-/-</sup> mice. This was reversed by Y27632 ( $p<0.05$ ,  $F_{3,28}=3.027$  locomotion x treatment). Data are shown as the total locomotor response following the injection of vehicle (vehicle), Ro 65-6570 or Y27632-Ro 65-6570 (Y27632-Ro 65) during the 45min test period. All data are shown as mean $\pm$ SEM, \*\*\* $p<0.001$  vs. other treatment groups.



**Fig. 7.  $\beta$ -arr1 regulation of GPCR function**

A.  $\beta$ -arr1<sup>+/+</sup>: In neurons containing  $\beta$ -arr1, the  $\delta$ OR agonist, SNC80, binds with  $\delta$ ORs and activates ROCK. As  $\beta$ -arr1 is associated with LIMK and one of the phosphatases, possibly SSL, within the trans-Golgi network (TGN), this activates cofilin to enhance actin filament severing and turnover and regulate  $\delta$ OR release to the cell membrane. B.  $\beta$ -arr1<sup>-/-</sup>. Without the inhibitory control of  $\beta$ -arr1, LIMK phosphorylates and inactivates cofilin. This leaves stable actin ‘tracks’ in place to allow unregulated but agonist-dependent  $\delta$ OR release to the plasma membrane in an agonist-dependent manner. C. Y27632. Preventing ROCK phosphorylation of LIMK by applying Y27632 prevents  $\delta$ OR activation of the pathway and agonist-induced release of  $\delta$ ORs to the cell membrane.