

Evidence that Behavioral Phenotypes of Morphine in β -arr2 $-/-$ Mice Are Due to the Unmasking of JNK Signaling

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The altered behavioral effects of morphine, but not most other mu agonists, in mice lacking β -arrestin 2, suggest that this scaffolding protein regulates the signaling cascade of this commonly used analgesic. One of the cascades that could be regulated by β -arrestin 2 is cJun-N-terminal kinase (JNK), which binds with β -arrestin 2 and modulates the analgesic effects of morphine. Using neurons lacking β -arrestin 2 (β -arr2 $-/-$) to examine this interaction, we found that β -arr2 $-/-$ neurons show altered intracellular distribution of JNK and cJun, and that morphine, but not fentanyl, increased the nuclear localization of the phosphorylated, therefore activated, form of cJun, a JNK target in dorsal root ganglia neurons. This suggests that deleting β -arrestin 2 affects the JNK cascade. We therefore examined whether some of the behavioral phenotypes of mice lacking β -arrestin 2 could be a result of altered JNK signaling. Indeed, two different JNK inhibitors reversed the enhanced analgesic effect of morphine, a known phenotype of β -arr2 $-/-$ mice, to $+/+$ levels. Both the reduced locomotor effect of morphine and the psychomotor sensitization to repeated morphine administration in β -arr2 $-/-$ mice were also returned to $+/+$ levels by inhibiting JNK. In contrast, the behavioral effects of fentanyl were neither genotype-dependent nor affected by JNK inhibition. Furthermore, a PKC inhibitor had a similar effect as inhibiting JNK in reducing the enhanced analgesic effect of morphine in β -arr2 $-/-$ mice to $+/+$ levels. In summary, removing β -arrestin 2 reveals mu receptor activation of the JNK cascade in a ligand-specific manner explaining several behavioral phenotypes of β -arr2 $-/-$ mice.

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INTRODUCTION

One of the many examples that the ubiquitously expressed arrestins serve as more than terminators of receptor signaling through initiating receptor internalization (Groer *et al*, 2011; Shenoy and Lefkowitz, 2011; Shukla *et al*, 2011) is the phenotype of mice lacking β -arrestin 2 (β -arr2 $-/-$; Bohn *et al*, 1999, 2002; Raehal and Bohn, 2011). As morphine does not induce significant internalization of the mu opioid receptor, the effects of morphine in β -arr2 $-/-$ mice cannot be explained by this prototypical role of β -arrestin 2. However, some progress has recently been made in revealing several arrestin-dependent, yet internalization-independent, signaling pathways of the mu opioid receptor that explain some, but not all, effects of morphine in mice

lacking β -arrestin 2 (Arttamangkul *et al*, 2008; Dang *et al*, 2011; Quillinan *et al*, 2011; Walwyn *et al*, 2007).

Observations of ligand-specific signaling, or the ability of different ligands to activate specific effectors, were first made in the 1980s (Gee and Yamamura, 1983), but only recently have become an accepted dimension of G-protein-coupled receptor (GPCR) signaling (Reiter *et al*, 2012; Urban *et al*, 2007). Such functional selectivity is believed to result from the ability of different agonists of the same GPCR to induce ligand-specific conformational changes of the receptor. These distinct conformations recruit different proteins to the agonist-bound receptor and activate downstream signaling cascades in an agonist-specific manner (Galandrin *et al*, 2008). The opioid family of GPCRs are no exception to such diversity; ligands of the mu, delta, and kappa opioid receptors are known to activate functionally distinct signaling cascades (Bruchas and Chavkin, 2010; Melief *et al*, 2010; Peng *et al*, 2009; Pradhan *et al*, 2010).

The enhanced analgesic effect of morphine but not many other mu agonists in mice lacking β -arrestin 2 suggests that morphine, in activating a signaling cascade that is regulated by β -arrestin 2, differs from other agonists (Bohn *et al*, 1999, 2002). As β -arrestin 2 is a multi-functional scaffolding

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and signal transduction protein (Xiao *et al*, 2007, 2010), the signaling cascade responsible for such ligand specificity remains unknown. However, the arrestins bind with, and regulate, many components of the map kinase cascade, one of which is the stress-activated protein kinase, cJun-N-terminal kinase (JNK), a kinase that has recently been shown to play an important role in ligand-directed signaling of the mu and kappa opioid receptors (Melief *et al*, 2010).

As JNK binds with β -arrestin 2, this scaffolding protein may modulate mu receptor activation of the JNK cascade in a ligand-specific manner. Using mice lacking β -arrestin 2 to determine how this scaffolding protein regulates mu receptor signaling, we have found that altered JNK activity explains some of the behavioral phenotypes of mice lacking β -arrestin 2.

MATERIALS AND METHODS

Animals

β -Arrestin 1 (also known as arrestin 2) or β -arrestin 2 (also known as arrestin 3) mice, fully back-crossed into the C57Bl/6 background, were kindly supplied by Dr Lefkowitz (HHMI, University of North Carolina, Chapel Hill, NC). The behavioral experiments used equal numbers of adult male and female β -arrestin (β -arr) 1/2 $+/+$ and $-/-$ mice that were 81 ± 10 days old, 22.3 ± 3.7 g in weight and bred from heterozygous matings. For the cellular experiments, early postnatal β -arr2 $+/+$ and $-/-$ pups were obtained from homozygous matings, one generation from heterozygous breeders. 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.

SDS-PAGE and Western Blot Analysis of JNK and Phospho-JNK Proteins Levels

Dorsal root ganglia (DRGs) were collected from all spinal levels of either β -arr2 $+/+$ or $-/-$ adult mice, weighed and homogenized in the following lysis buffer; 20 mM Tris-HCl pH 7.4, 0.32 M sucrose, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 1 mM $\text{Na}_4\text{P}_2\text{O}_7$, $1 \times$ Halt Protease and Phosphatase Inhibitor Cocktail (Pierce, Thermo Scientific, Rockford, IL), and protein content determined (BCA, Pierce). Protein samples (50 $\mu\text{g}/\text{sample}$) were separated by SDS-PAGE (10% NuPAGE gel; Invitrogen, Carlsbad, CA) and transferred to an Immobilon PVDF membrane (0.45 μm ; Millipore, Billerica, MA). After several washes in TBS and a 120 min blocking step (Casein/0.1% Tween-20, Thermo Scientific), the membrane was incubated in anti-JNK or anti-phospho-JNK at 1:100 (Thr183/Tyr185), from both Cell Signaling (Danvers, MA) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 2.5×10^{-2} $\mu\text{g}/\text{ml}$, Thermo Scientific), o/n at 4 °C. The membrane was then washed in TBS with 0.1% Tween-20, incubated in Stabilized Peroxidase-conjugated secondary antibodies, anti-rabbit IgG for JNK (1:100) or anti-mouse IgG for phospho-JNK (1:100), or GAPDH (1:10 000) for 120 min at RT (Pierce, Rockford, IL). The labeled protein bands were visualized by chemiluminescence (Super signal Chemiluminescent Substrate, Pierce), and signal intensity (pixels/ mm^2) quantified (ImageJ, NIH).

For each sample, the signal intensity of JNK or phospho-JNK was normalized to that of GAPDH. *Statistical analysis:* The mean of the three experiments for each of the six samples are expressed as the mean \pm SEM and the differences between groups determined by one-way ANOVA (Analyse-it Software, Leeds, UK) with significance accepted at $p < 0.05$.

Cell Culture

DRGs from all spinal levels were harvested from early postnatal (p0–1) β -arr2 $+/+$ or $-/-$ pups. After chemical dissociation in trypsin (Invitrogen) and physical dissociation by trituration, 1×10^4 cells were plated on poly-D-lysine (Sigma, St Louis, MO) and laminin (Invitrogen) coated coverslips of MatTek dishes (MatTek, Ashland, MA) as previously described (Walwyn *et al*, 2007). The cells were maintained for 24–48 h in Neurobasal/B27 media containing NGFs (10 ng/ml) *in vitro* after which the cells were treated and fixed for immunocytochemistry or used to determine mu agonist inhibition of voltage-dependent Ca^{2+} channels (VDCCs).

Immunocytochemistry

β -arr2 $+/+$ or $-/-$ cells remained untreated or were treated with morphine (NIDA) or fentanyl (Sigma) for 10 min, washed and fixed. After a series of washes, a 0.3% Triton X100 permeabilization step, and a 10% serum blocking step, anti-JNK3 (1:500, Santa Cruz Biotechnology, Santa Cruz, CA) or anti-phosphorylated JNK (anti-phospho-JNK: 1:100, Cell Signaling) were added. In a second, matching, set of plates, primary antibodies against cJun (1:500, Santa Cruz Biotechnology) and phosphorylated cJun (anti-phospho-cJun, 1:250, Santa Cruz Biotechnology) were added. After o/n incubation and further washes, Alexa-555-conjugated donkey anti-goat/mouse (1:1000 for phospho-cJun and phospho-JNK; Invitrogen) and Alexa-488-conjugated donkey anti-rabbit/goat (1:1000 for cJun and 1:400 for JNK; Invitrogen) were added to each plate for 90 min at RT. After a final series of washes, the cells were mounted in Prolong containing 4',6-diamidino-2-phenylindole (DAPI; Invitrogen). Medium-sized DRGs (20–30 μm) were visualized by a $\times 60$ oil immersion objective on a Nikon TE2000 microscope and images were captured by a Retiga 1300Exi CCD camera (QImaging, British Columbia, Canada). All cells were imaged at the mid-nuclear level using DAPI as a guide. Pixel intensity per unit area for each of the three channels (RGB) was quantified by IPLab (version 3.6.5, BioVision Technologies, Exton, PA). *Statistical analysis:* Data from untreated cells were expressed as mean \pm SEM of the nuclear/cytosolic signal intensity for > 100 cells/sample. The effect of different mu agonists on cJun and phospho-cJun localization was determined by comparing the percent change of the nuclear:cytosolic ratio in the agonist treated *vs* untreated cells. Differences between groups were determined using two-way ANOVA between groups (Analyse-it Software) and significance accepted at $p < 0.05$.

Analgesia

Thermal nociception was measured by the warm-water tail-immersion assay in which the latency to remove the tail

from a 49.5 °C water bath was measured. After a basal response was obtained (average of three to four tests), one of the JNK or PKC inhibitors, or matching vehicle, was injected, another basal measurement was obtained 30 (PKC inhibitor) or 60 (JNK inhibitor) min later and then morphine or fentanyl was administered. The JNK inhibitors used were: SP600125 (SP6; Anthra[1,9-*cd*]pyrazol-6(2*H*)-one, 1,9-pyrazoloanthrone, 30 mg/kg i.p., EMD Chemicals, Merck KGaA, Darmstadt, Germany) or BI78D3 (4-(2,3-Dihydrobenzo[*b*][1,4]dioxin-6-yl)-5-(5-nitrothiazol-2-ylthio)-4*H*-1,2,4-triazol-3-ol) 25 mg/kg i.p., EMD Chemicals). Both compounds inhibit the activation of all JNK isoforms by binding with either the ATP docking site (SP6; Shin *et al*, 2002) or the D-domain consensus sequence in JNK to inhibit the docking of upstream kinases (BI78D3; Stebbins *et al*, 2008). The PKC inhibitor used, Bisindolylmaleimide VIII (Ro-31-7549, EMD Chemicals), inhibits PKC α , β I, β II, γ , ϵ (Wilkinson *et al*, 1993). *Statistical analysis*: Data are expressed as the response in seconds (s), and as the mean \pm SEM of each group. Differences between groups were determined by one- or two-way ANOVA with repeated measures and factorial analysis at each time interval using Stat View (v5). Significance was accepted at $p < 0.05$.

Locomotion

Locomotor activity was measured in 16.7 \times 12.7 cm² boxes by an infrared video-camera and Ethovision video tracking software (v7.1; Noldus, Tacoma). The mice were habituated for 15 min on the first day and, on each of the following 3 days, were given SP6 (30 mg/kg i.p.), or vehicle, 60 min before morphine (10 mg/kg s.c.) or fentanyl (0.2 mg/kg s.c.) and placed in the locomotor boxes. Locomotion was assessed over the following 30 min. *Statistical analysis*: Data are expressed as mean \pm SEM and differences between groups determined by one- or two-way ANOVA with repeated measures and factorial analysis at each time interval (Stat View).

Electrophysiology

Voltage-dependent Ca²⁺ currents were recorded from small-medium-sized DRG neurons 20–30 μ m in diameter under whole-cell voltage-clamp conditions as previously described (Tan *et al*, 2003; Walwyn *et al*, 2007). Cells were perfused with an external solution containing 10 mM CaCl₂, 130 mM tetraethylammonium chloride, 5 mM HEPES, 25 mM *d*-glucose and 0.25 μ M tetrodotoxin at pH 7.35. The patch electrode was filled with an internal solution composed of 105 mM CsCl, 40 mM HEPES, 5 mM *d*-glucose, 2.5 mM MgCl₂, 10 mM EGTA, 2 mM Mg-ATP, and 0.5 mM GTP at pH 7.2. Ca²⁺ currents were evoked every 10 s by 40-ms voltage steps from –80 to +10 mV using an Axopatch 200B patch-clamp amplifier. Capacitance and series resistance were corrected with the compensation circuitry on the amplifier. Series resistance was compensated by 80–90%. Leak currents were subtracted using a P/6 protocol. Recorded signals were acquired and analyzed using Axon pClamp v8.0 or 9.0 software (Axon Instruments, Foster City, CA). *Drug application and desensitization protocols*: The JNK inhibitor, SP6, was dissolved in DMSO as a 10-mM stock solution and diluted with the culture medium to a final

concentration of 10 μ M for application. The effect of SP6 on mu receptor–VDCC inhibition was assessed by a 4-h pre-incubation with SP6 (10 μ M). For the desensitization experiments, β -arr2+/+ and –/– DRG neurons were pre-incubated with 1 μ M morphine, D-Ala₂-MePhe₄-Glyol₅ Enkephalin (DAMGO), or fentanyl, in the presence or absence of 10 μ M SP6 for 4 h, the media removed, and the cells were washed by an \sim 100 \times solution exchange before being challenged by the full agonist, DAMGO (1 μ M). *Statistical analysis*: Mean Ca²⁺ current amplitudes were measured (pCLAMP 9.0; Axon Instruments, CA) between 5 and 10 ms after initiating the depolarizing step. Mean current amplitudes were then plotted against time. Stable recordings were fitted by a linear function to compare, by extrapolation, control current amplitude to the current amplitude recorded in the presence of opioid receptor agonists or SP6. Data are expressed as mean \pm SEM and were compared using ANOVA with a *post hoc* Tukey's test with significance accepted at $p < 0.05$. Recordings that exhibited marked rundown (> 30%) were discarded as were significant outliers, five out of the \sim 300+ recordings (Prism v5.03).

RESULTS

DRG Neurons Lacking β -Arrestin 2 Show Altered JNK and cJun Localization

JNK and phospho-JNK expression. As β -arrestin 2 facilitates GPCR activation of the JNK cascade (Willoughby and Collins, 2005), genetically deleting β -arrestin 2 may result in a compensatory adaptation in the expression of JNK and phospho-JNK. This was examined by SDS-PAGE and western blotting in β -arr2–/– and +/+ adult DRGs. No effect of genotype was found. The relative intensities of the protein of interest normalized to that of GAPDH were: JNK; 48 kDa: β -arr2+/+; 0.39 \pm 0.05, β -arr2–/–: 0.33 \pm 0.05. 54 kDa: β -arr2+/+; 0.23 \pm 0.06, β -arr2–/–: 0.16 \pm 0.04. phospho-JNK; 54 kDa: β -arr2+/+; 0.61 \pm 0.13, β -arr2–/–: 0.64 \pm 0.04.

The basal intracellular location of JNK and cJun. As β -arrestin 2 contains a nuclear export sequence resulting in its constitutive exclusion from the nucleus (Song *et al*, 2006), β -arr2–/– neurons may show altered nuclear or cytosolic localization of proteins, such as JNK, that bind with β -arrestin 2. We therefore examined the effect of deleting β -arrestin 2 on the relative nuclear:cytosolic ratio of JNK, phospho-JNK, and the major JNK target, cJun, and phospho-cJun. β -arr2–/– DRG neurons showed a basal increase in the nuclear localization of JNK, phospho-JNK, and cJun, but not phospho-cJun (Figure 1b).

Ligand-induced changes in phospho-cJun location. We next examined whether two mu agonists, morphine and fentanyl, previously shown to activate the JNK cascade (Melief *et al*, 2010) could alter the intracellular location of phospho-cJun in β -arr2+/+ and –/– neurons. We focused on phospho-cJun for a number of reasons; the activity of this terminal kinase of the JNK pathway is finely regulated by JNK (Hibi *et al*, 1993) and, importantly, the basal location of phospho-cJun was unaffected by deleting β -arrestin 2. We found that morphine increased the relative

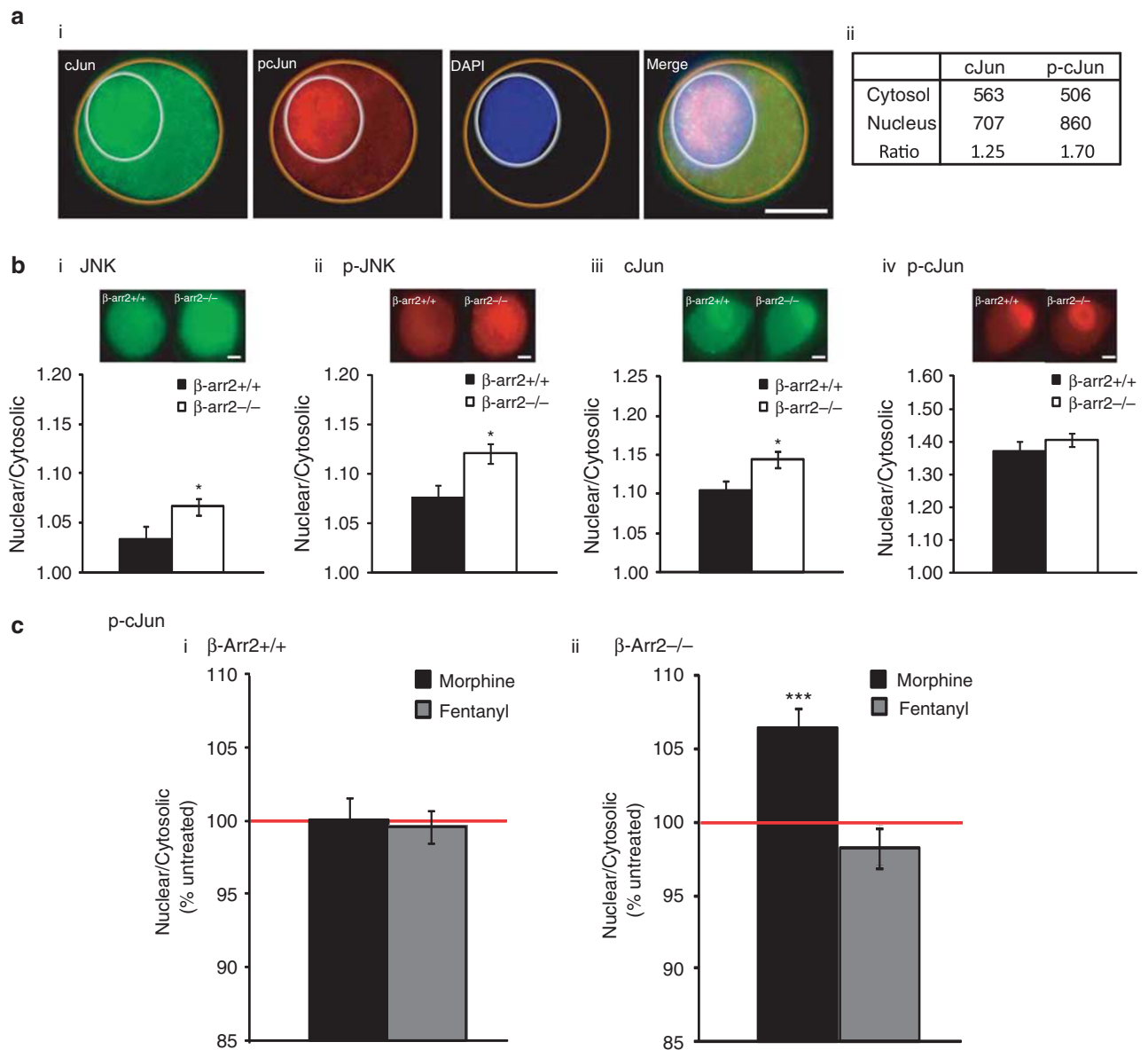


Figure 1 DRG neurons as a model to examine the JNK cascade. DRG neurons from early postnatal mice were used to examine the effect of deleting β -arrestin 2 and different mu receptor agonists on the relative nuclear:cytosolic ratio of JNK and cJun. (a) The relative pixel intensity/unit area of the nuclear or cytosolic labeling for JNK, phospho-JNK, cJun, or phospho-cJun was imaged and quantified in individual neurons. An example of the labeling and quantification for one cell are shown in (ai, aii), respectively. (b) This method was used to quantify the nuclear:cytosolic ratio of JNK, phospho-JNK, cJun, and phospho-cJun, in untreated β -arr2+/+ vs β -arr2-/- neurons ($n > 100$ neurons/sample). β -arr2-/- neurons showed a relative increase in the nuclear localization of JNK, phospho-JNK, and cJun, ($*p < 0.05$ vs β -arr2+/+), but not phospho-cJun ($p = 0.75$). (c) While morphine treatment ($1 \mu\text{M}$, 10 min) did not affect the nuclear:cytosolic ratio of phospho-cJun in β -arr2+/+ neurons, this mu agonist did increase phospho-cJun in the nucleus vs cytosol of β -arr2-/- neurons. $***p < 0.001$ vs untreated. Scale bars = $10 \mu\text{m}$.

intensity of phospho-cJun in the nucleus of β -arr2-/- but had no effect in +/+ neurons. Fentanyl did not alter phospho-cJun localization in +/+ or -/- neurons (Figure 1c). Neither ligand altered the nuclear:cytosolic ratio of cJun (data not shown).

JNK Inhibition Reverses Behavioral Phenotypes of Morphine in β -arr2-/- Mice

Using DRG neurons as a model, we have found altered basal and mu agonist-induced changes in the intracellular

location of JNK and/or cJun, suggesting that deleting β -arrestin 2 alters aspects of the JNK cascade. As this cascade can modulate the behavioral effects of mu agonists (Chen *et al*, 2008; Melief *et al*, 2010), we next examined whether the behavioral phenotypes of β -arr2-/- mice could be a result of altered JNK activity.

Analgesia. The role of JNK in thermal analgesia was examined by administering one of two JNK inhibitors, SP6 or BI78D3, to β -arr2-/- and +/+ mice, 60 min before morphine (5 and 10 mg/kg s.c.) or fentanyl (0.2 mg/kg s.c.).

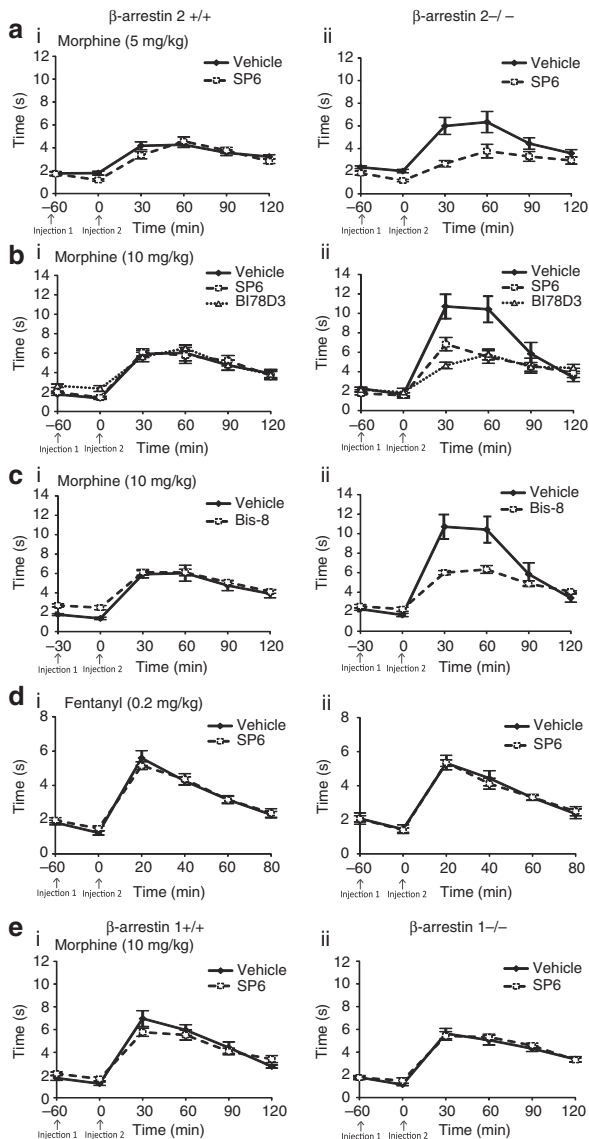


Figure 2 The enhanced thermal analgesic effect of morphine in β -arr2 $^{-/-}$ mice is reduced to $+/+$ levels by inhibiting JNK or PKC. The warm-water (49.5°C) tail-immersion assay was used to assess the analgesic effects of different mu agonists in β -Arr2 $+/+$ and $^{-/-}$ mice and the resultant data are presented as the response (s; y axis) over time (min; x axis). (a, b) β -Arr2 $^{-/-}$ mice showed a dose-dependent increase in the thermal analgesic effects of morphine ((a) 5 and (b) 10 mg/kg morphine s.c., 5 mg/kg; $p = 0.12$ vs β -arr2 $+/+$, $F_{1,5} = 1.773$; 10 mg/kg; $p < 0.001$ vs β -arr2 $+/+$, $F_{1,5} = 13.227$), which was reversed by the JNK inhibitor, SP6 (aii) 5 mg/kg; $p < 0.05$ vs vehicle, $F_{1,5} = 2.422$; (bii) 10 mg/kg; $p < 0.01$ vs vehicle, $F_{1,5} = 4.501$). BI78D3 (bii), similarly reversed the enhanced analgesia seen in morphine-treated (10 mg/kg s.c.) β -arr2 $^{-/-}$ mice ($p < 0.001$ vs vehicle, $F_{1,5} = 6.142$). Neither JNK inhibitor altered the β -arr2 $+/+$ response ((ai) 5 mg/kg; $p = 0.06$ vs vehicle, $F_{1,5} = 10.74$; (aii) 10 mg/kg; SP6: $p = 0.94$ vs vehicle, $F_{1,5} = 0.457$; BI78D3: $p = 0.71$ vs vehicle, $F_{1,5} = 0.575$). (c) The PKC inhibitor Bisindolylmaleimide VIII (10 mg/kg i.p.) also reversed the enhanced analgesia seen after morphine administration (10 mg/kg s.c.) in β -arr2 $^{-/-}$ mice ($p < 0.001$ vs vehicle, $F_{1,5} = 7.099$), while having no effect in β -arr2 $+/+$ mice ($p = 0.76$ vs vehicle, $F_{1,5} = 0.937$). (d) Mice treated with fentanyl (0.2 mg/kg s.c.) showed no effect of the β -arrestin 2 deletion ($p = 0.84$ vs β -arr2 $+/+$, $F_{1,5} = 0.403$) or JNK inhibition (β -arr2 $+/+$: $p = 0.59$ vs vehicle, $F_{1,5} = 0.748$; β -arr2 $^{-/-}$: $p = 0.96$ vs vehicle, $F_{1,5} = 0.194$). (e) There was also no effect of deleting β -arrestin 1 ($p = 0.10$ vs β -arr2 $+/+$, $F_{1,5} = 1.883$) or JNK inhibition on the morphine response of β -arr1 $^{-/-}$ or $+/+$ mice (SP6: β -arr1 $+/+$: $p = 0.07$ vs vehicle, $F_{1,5} = 2.089$; β -arr1 $^{-/-}$: $p = 0.80$ vs vehicle, $F_{1,5} = 0.224$). Injection 1 = JNK or PKC inhibitor, or matching vehicle. Injection 2 = morphine or fentanyl.

Thermal analgesia was assessed by the warm-water tail-immersion assay 20–30 min later and at regular intervals thereafter. In β -arr2 $+/+$ mice, neither of the JNK inhibitors altered the analgesic profile of morphine, which showed a comparable dose-response curve to previous publications (Sora et al, 1997; Bohn et al, 2000). In contrast, both SP6 and BI78D3, reversed the enhanced analgesic effect of morphine in β -arr2 $^{-/-}$ mice to wild-type levels (Figure 2a and b). The analgesia induced by fentanyl was neither genotype-dependent nor influenced by JNK. In addition, the higher basal analgesia of β -arr2 $^{-/-}$ (2.19 ± 0.05 s) vs $+/+$ (2.03 ± 0.05 s, $p < 0.05$) mice was not affected by either SP6 or BI78D3. As PKC has been shown to activate JNK (Lopez-Bergami et al, 2005; Melief et al, 2010), we next examined whether the effect of morphine in β -arr2 $^{-/-}$ mice was also PKC-dependent. The PKC inhibitor, Bisindolylmaleimide VIII, reversed the enhanced effect of morphine in β -arr2 $^{-/-}$ mice to wild-type levels (Figure 2c). We also examined whether β -arrestin 1 may be responsible for the effect of JNK in β -arr2 $^{-/-}$ mice but found no effect of deleting β -arrestin 1 or inhibiting JNK in the analgesic response to morphine in these mice (Figure 2e).

Psychomotor effects of morphine

Acute locomotion: Another of the behavioral phenotypes of β -arr2 $^{-/-}$ mice is the reduced locomotor effect of morphine (Bohn et al, 2003; Urs et al, 2011). We hypothesized that, similar to the analgesic effect of morphine, this may also be due to altered JNK activity. The JNK inhibitor, SP6, or vehicle, was therefore administered 60 min before assessing the locomotor effects of morphine or fentanyl in β -arr2 $+/+$ and $^{-/-}$ mice. SP6 had no effect in β -arr2 $+/+$ mice, but SP6-treated $^{-/-}$ mice showed an enhanced locomotor response to morphine matching that of β -arr2 $+/+$ mice (Figure 3a). By contrast, the locomotor effect of fentanyl was neither genotype nor SP6-dependent (Figure 3b).

Psychomotor sensitization: As the locomotor effect of a single morphine injection was influenced by both genotype and JNK inhibition, locomotor sensitization induced by repeated morphine administration could similarly be modulated by JNK in β -arr2 $^{-/-}$ mice. We found that 3 days of repeated morphine administration resulted in less locomotor sensitization in β -arr2 $^{-/-}$ than $+/+$ mice (10 mg/kg; Figure 4a). This was reversed by SP6, administered 60 min before morphine on each of the 3 test days (Figure 4b). The locomotor sensitization induced by repeated fentanyl administration was not influenced by deleting β -arrestin 2 or inhibiting JNK (Figure 4c and d).

Possible cellular targets of the JNK cascade. Inhibiting JNK reduced the analgesic efficacy of morphine 30 min after this drug was administered. This suggests that JNK phosphorylation of cytosolic targets, rather than the transcriptional activity of cJun, modulates morphine analgesia. We therefore examined whether opioid coupling with VDCCs, a G $\beta\gamma$ -mediated second messenger cascade, may be influenced by JNK in a ligand-specific manner. We examined both the acute effect of inhibiting JNK on mu receptor-VDCC coupling as well as desensitization of opioid

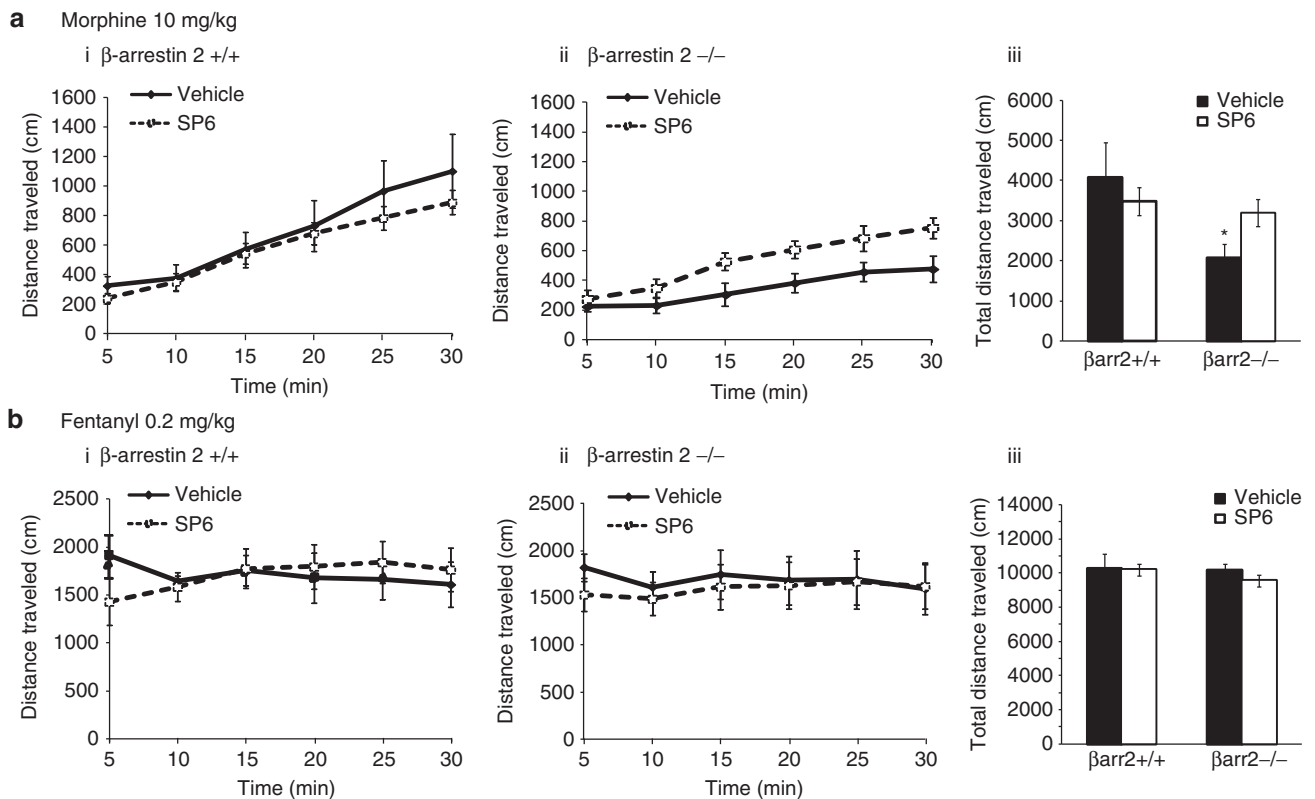


Figure 3 The reduced locomotor effect of morphine in β -arr2^{-/-} mice is returned to ^{+/+} levels by inhibiting JNK. The locomotor effect of different mu agonists in β -arr2^{+/+} and ^{-/-} mice is shown over the duration of the 30-min test in the line graphs with distance traveled (cm; y axis) shown over time (x axis). The total distance traveled (cm; y axis) is shown in the accompanying bar graph. (a) The locomotor effect of morphine was less in β -arr2^{-/-} mice compared with their wild-type littermates ($p < 0.01$ vs β -arr2^{+/+}, $F_{1,11} = 14.251$). SP6 (30 mg/kg i.p.) increased the effect of morphine in β -arr2^{-/-} mice ($p < 0.001$ vs vehicle, $F_{1,11} = 19.835$) to ^{+/+} levels ($p = 0.52$ vs β -arr2^{+/+}, $F_{1,14} = 0.429$) but had no effect in β -arr2^{+/+} mice ($p = 0.46$ vs vehicle, $F_{1,14} = 0.577$). (b) Mice treated with fentanyl (0.2 mg/kg s.c.) showed no effect of genotype ($p = 0.81$ vs β -arr2^{+/+}, $F_{1,9} = 0.061$) or JNK inhibition (SP6: β -arr2^{+/+}: $p = 0.68$ vs vehicle, $F_{1,12} = 0.169$; β -arr2^{-/-}: $p = 0.16$ vs Vehicle, $F_{1,11} = 2.194$).

receptor-VDCC inhibition following 4h of opiate pre-treatment. We found no effect of SP6 pre-treatment on the acute VDCC inhibition induced by morphine, fentanyl or DAMGO in either β -arr2^{+/+} or ^{-/-} neurons (Table 1). However, desensitization induced by each of the ligands revealed both genotype and JNK effects, as shown by a 1- μ M test dose of DAMGO. In β -arr2^{+/+} neurons, the desensitization induced by morphine (4h, 1 μ M) was prevented by SP6 co-incubation. In β -arr2^{-/-} neurons, morphine did not induce significant desensitization, and this was unaffected by SP6 co-incubation. Pre-incubation with either DAMGO or fentanyl resulted in significant desensitization in both β -arr2^{+/+} and ^{-/-} neurons that was not altered by SP6.

DISCUSSION

When compared with other mu agonists, morphine induces less internalization of the mu opioid receptor (Borgland *et al*, 2003; McPherson *et al*, 2010; Zheng *et al*, 2011). This suggests that the prototypical role of β -arrestin 2 in initiating receptor internalization does not explain how the effects of morphine, but not of other mu agonists, are altered in mice lacking β -arrestin 2. Here, we demonstrate that morphine activation of JNK in the absence of β -arrestin

2 explains ligand-specific behavioral phenotypes of β -arr2^{-/-} mice. These findings also demonstrate that the JNK cascade may be activated in a ligand-specific manner, providing further evidence of the role of this member of the map kinase pathway in mediating ligand-directed signaling of the mu receptor.

Compared with other mu agonists such as fentanyl, the relative inability of morphine to cause significant receptor phosphorylation, results in the recruitment of PKC to morphine, but not fentanyl, bound mu receptors (Zheng *et al*, 2011). As PKC is known to both scaffold with and phosphorylate JNK (Lopez-Bergami *et al*, 2005; Ping, 2003; Pontrelli *et al*, 2004), we propose that in wild-type mice, morphine recruitment of PKC activates JNK but that β -arrestin 2 recruitment limits further JNK activation. However, if β -arrestin 2 is absent and is coupled with the inability of morphine to recruit β -arrestin 1 (Groer *et al*, 2011), sufficient PKC recruitment results in sustained activation of the JNK cascade in β -arr2^{-/-} neurons to affect analgesia. In contrast, other mu agonists, such as fentanyl differ from morphine as they are able to phosphorylate the mu receptor and recruit either β -arrestin 1 or 2 (Groer *et al*, 2011; Zheng *et al*, 2011). In the absence of β -arrestin 2, these agonists recruit β -arrestin 1, PKC is not recruited and the JNK cascade is not significantly activated.

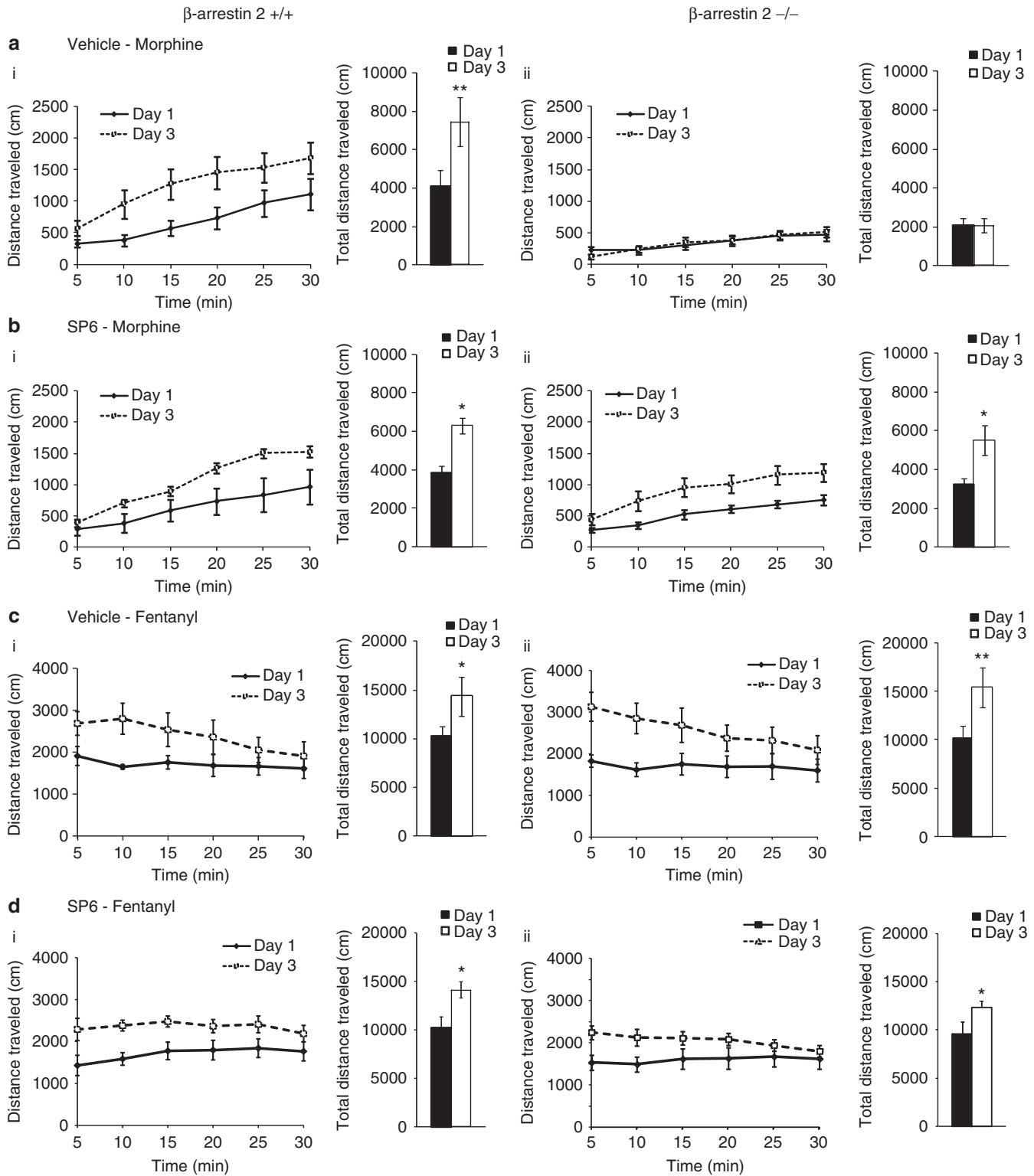


Figure 4 The reduced psychomotor effect of morphine in β -arr2 $-/-$ mice is similarly returned to $+/+$ levels by inhibiting JNK. These data are presented in the same format as in Figure 2 showing both the distance traveled over time in the line graphs and total locomotion in the accompanying bar graphs (ai) A 3-day protocol of successive morphine (10 mg/kg s.c.) treatment of β -arr2 $+/+$ mice resulted in an enhanced locomotor response to the same dose of morphine by day 3 ($p < 0.01$ day 1 vs day 3, $F_{1,5} = 16.972$). (aii) Conversely, similarly treated β -arr2 $-/-$ mice showed no such increase ($p = 0.41$ day 1 vs day 3, $F_{1,6} = 0.765$). (bi) Administration of SP6 (30 mg/kg i.p.), 60 min before each morphine injection had no effect on the locomotor sensitization in β -arr2 $+/+$ mice ($p < 0.05$ day 1 vs day 3, $F_{1,9} = 6.866$) but reversed the lack of sensitization in the β -arr2 $-/-$ mice ($p < 0.05$ day 1 vs day 3, $F_{1,5} = 6.989$) to wild-type levels. (c, d) Mice treated with fentanyl (0.2 mg/kg s.c.) showed locomotor sensitization ($p < 0.001$ day 1 vs day 3, $F_{1,23} = 63.235$), that was unaffected by β -arrestin 2 deletion or SP6 administration (vehicle; $p = 0.93$ day \times genotype, $F_{1,23} = 0.007$, SP6; $p = 0.25$ day \times genotype \times injection, $F_{1,23} = 1.382$). * $p < 0.05$, ** $p < 0.01$.

Table 1 JNK Inhibition by SP6 has no Effect on Mu Receptor–VDCC Inhibition in β -arr2 $^{-/-}$ or $+/+$ Neurons

Agonist Pre-treatment	Morphine		Fentanyl		DAMGO	
	–	SP6	–	SP6	–	SP6
β -arr2 $+/+$	32.5 \pm 4.1	37.8 \pm 5.2	36.3 \pm 2.4	36.5 \pm 2.5	44.4 \pm 2.9	56.0 \pm 7.2
β -arr2 $-/-$	22.0 \pm 3.6*	23.6 \pm 2.8*	26.4 \pm 3.1*	24.4 \pm 4.4*	26.9 \pm 3.0**	23.1 \pm 4.4**

The effect of SP6 incubation (10 μ M, 4 h) on Ca²⁺ channel inhibition (%) by the mu opioid receptor agonists, morphine, fentanyl, and DAMGO (1 μ M each) was assessed in early postnatal dorsal root ganglia neurons from β -arr2 $+/+$ and $-/-$ mice.

*, ** $p < 0.05$, 0.01 vs β -arr2 $+/+$, same treatment.

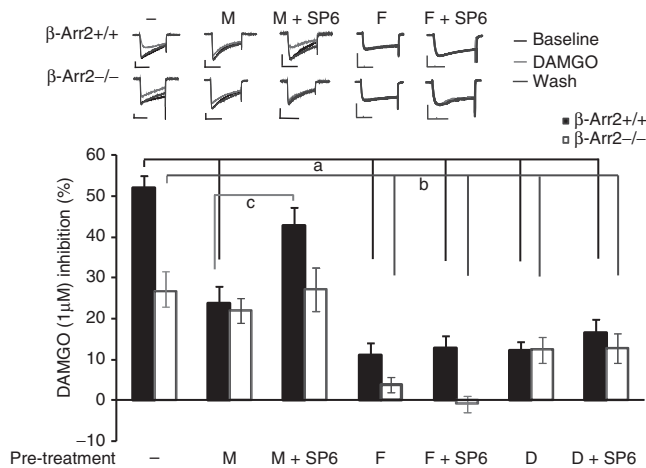


Figure 5 Mu receptor–Ca²⁺ channel inhibition as a possible cytosolic JNK target. Mu receptor inhibition of voltage-dependent Ca²⁺ channels (VDCC) was examined in β -arr2 $+/+$ and $-/-$ DRG neurons. Exemplar currents are shown above the bar graph, which summarizes the effect of mu agonist pre-treatment coupled with JNK inhibition on mu receptor–VDCC inhibition, $n = 6$ –30 neurons/sample. Although JNK inhibition had no effect on mu receptor–VDCC inhibition in β -arr2 $-/-$ or $+/+$ neurons (Table 1), the desensitization induced by a 4-h pre-treatment of β -arr2 $+/+$ neurons with morphine (1 μ M) was prevented by SP6 co-incubation (10 μ M, 4 h). In contrast, β -arr2 $-/-$ neurons showed no desensitization to morphine and no effect of SP6. Both fentanyl and DAMGO pre-treatment desensitized mu receptor–VDCC inhibition in β -arr2 $+/+$ and $-/-$ neurons but this was not affected by SP6 co-incubation. M = morphine; F = fentanyl; D = DAMGO. a: β -arr2 $+/+$ neurons; $p < 0.05$ vs M, $p < 0.001$ vs F, F + SP6, D, D + SP6. b: β -arr2 $-/-$ neurons; $p < 0.05$ vs D, D + SP6, $p < 0.001$ vs F, F + SP6. c: β -arr2 $+/+$ neurons; $p < 0.05$ vs M + SP. Scale bars = 40 ms on the x axis and 0.4 nA on the y axis.

β -Arrestin 2 has been shown to bind with JNK3, but not JNK1 or 2, to control GPCR activation of the JNK cascade (McDonald *et al*, 2000; Miller *et al*, 2001; Song *et al*, 2006, 2009; Willoughby and Collins, 2005). Removing β -arrestin 2 may therefore alter both the location and control of JNK3 to allow sustained JNK activation in β -arr2 $-/-$ mice. If JNK3 were indeed involved, this is a different isoform to the JNK2-dependent regulation of receptor uncoupling following repeated morphine administration (Melief *et al*, 2010). In our experiments, acute mu receptor coupling with VDCCs was unaffected by JNK inhibition, suggesting that neither the mu receptor nor $G\beta\gamma$ -coupled Ca²⁺ channels are downstream targets of the JNK activity unmasked by deleting β -arrestin 2. In contrast, SP6 modulation of mu receptor–VDCC desensitization in β -arr2 $+/+$ neurons shows a JNK dependency (Figure 5) that could be involved

in the JNK2 regulation of mu receptor–effector coupling and morphine desensitization *in vivo* (Melief *et al*, 2010). This raises an interesting hypothesis that the same isoform, or possibly different JNK isoforms, may regulate different signaling cascades of the mu receptor depending on whether β -arrestin 2 is present or absent and the protocol of morphine application.

The effects of morphine on locomotion and subsequent sensitization in rodents are considered a model of the initial phase of addiction in humans (Robinson and Berridge, 1993; Vanderschuren and Pierce, 2011). This response requires D1 receptors, the phosphoprotein DARPP32 (Becker *et al*, 2001; Borgkvist *et al*, 2007; Urs *et al*, 2011) and ERK (Borgkvist *et al*, 2008; Urs *et al*, 2011). We, and others, have shown that the locomotor effect of morphine is blunted in mice lacking β -arrestin 2 (Bohn *et al*, 2003; Urs *et al*, 2011). As morphine induces the formation of a β -arrestin 2/ERK complex in D1 neurons, it has been suggested that this complex mediates the hyperlocomotor effect of morphine (Urs *et al*, 2011). In using pharmacological tools to inhibit JNK in β -arrestin 2 $-/-$ mice, we have shown that activation of the JNK cascade in the absence of β -arrestin 2 explains the reduced locomotor response of morphine in these mice. Taken together, these data and those of Caron and colleagues (Urs *et al*, 2011) suggest that β -arrestin 2 plays complementary roles in this paradigm, one prevents JNK activation whereas the other allows a β -arrestin/ERK complex to form in D1 neurons. We propose that both roles synergize, and are necessary for the characteristic locomotor effect of morphine to occur.

Although others have found that sensitization to morphine is not β -arrestin 2-dependent (Bohn *et al*, 2003), we have found that this context-specific response to multiple doses of morphine is reduced in mice lacking β -arrestin 2. However, our assay differed in several important ways; we examined the first 30 min after morphine (30 vs 120 min), after 3 rather than 9 days of repeated morphine, and our sensitization protocol was context-associated. The initial loss of such ‘short-term’ sensitization in β -arr2 $-/-$ mice may therefore not reflect a lack of, but rather an attenuation of the onset of sensitization that may not be detectable in a context-independent manner after an extended protocol of morphine administration. Furthermore, as JNK inhibition recovered the locomotor response at similar relative proportions for each day of the test, this suggests that the JNK cascade does not block morphine sensitization. However, inhibiting JNK allows an initial locomotor response to morphine thus permitting sensitization to develop.

Our findings, and those of several others, show that β -arrestin 2 regulates mu receptor function through several different pathways. For example, the enhanced basal analgesia seen in β -arr2 $^{-/-}$ mice results from an enhanced level of constitutively active mu receptors (Lam *et al*, 2011; Walwyn *et al*, 2007). Such constitutive activity also explains the reduced VDCC inhibition by mu agonists in β -arr2 $^{-/-}$ neurons (Table 1; Figure 5; Lam *et al*, 2011; Walwyn *et al*, 2007). The well-known attenuated morphine desensitization and tolerance of the mu receptor in β -arr2 $^{-/-}$ mice may result from β -arrestin 2 inhibition of mu receptor resensitization, independent of receptor internalization (Dang *et al*, 2011; Quillinan *et al*, 2011). This could explain the lack of desensitization of DAMGO-VDCC inhibition following chronic morphine treatments in β -arr2 $^{-/-}$ neurons seen in Figure 5. Our data add yet another mechanism to β -arrestin 2 regulation of mu receptor function. We show that the enhanced morphine-induced analgesia and decreased locomotor response in β -arr2 $^{-/-}$ mice is JNK-mediated, demonstrating how β -arrestin 2 normally controls this pathway. It would be of further interest to examine whether this role of β -arrestin 2 in masking JNK activation may underlie the enhanced reward, attenuated tolerance, or indeed other pertinent phenotypes of morphine in mice lacking β -arrestin 2.

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DISCLOSURE

The authors declare no conflict of interest.

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