Src Kinase Inhibition Attenuates Morphine Tolerance without Affecting Reinforcement or Psychomotor Stimulation

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ABSTRACT

Background: Prolonged opioid administration leads to tolerance characterized by reduced analgesic potency. Pain management is additionally compromised by the hedonic effects of opioids, the cause of their misuse. The multifunctional protein β-arrestin2 regulates the hedonic effects of morphine and participates in tolerance. These actions might reflect μ opioid receptor up-regulation through reduced endocytosis. β-Arrestin2 also recruits kinases to μ receptors. We explored the role of Src kinase in morphine analgesic tolerance, locomotor stimulation, and reinforcement in C57BL/6 mice.

Methods: Analgesic (tail withdrawal latency; percentage of maximum possible effect, n = 8 to 16), locomotor (distance traveled, n = 7 to 8), and reinforcing (conditioned place preference, n = 7 to 8) effects of morphine were compared in wild-type, $\mu^{+/-}$, $\mu^{-/-}$, and β-arrestin2$^{-/-}$ mice. The influence of c-Src inhibitors dasatinib (n = 8) and PP2 (n = 12) was examined.

Results: Analgesia in morphine-treated wild-type mice exhibited tolerance, declining by day 10 to a median of 62% maximum possible effect (interquartile range, 29 to 92%). Tolerance was absent from mice receiving dasatinib. Tolerance was enhanced in $\mu^{+/-}$ mice (34% maximum possible effect; interquartile range, 5 to 52% on day 5); dasatinib attenuated tolerance (100% maximum possible effect; interquartile range, 68 to 100%), as did PP2 (91% maximum possible effect; interquartile range, 78 to 100%). By contrast, c-Src inhibition affected neither morphine-evoked locomotor stimulation nor reinforcement. Remarkably, dasatinib not only attenuated tolerance but also reversed established tolerance in $\mu^{+/-}$ mice.

Conclusions: The ability of c-Src inhibitors to inhibit tolerance, thereby restoring analgesia, without altering the hedonic effect of morphine, makes c-Src inhibitors promising candidates as adjuncts to opioid analgesics. **(Anesthesiology 2017; 127:878-89)**

ALTHOUGH estimates vary, 9% of Americans and 19% of Europeans (11 to 55% in developing countries) are reported to experience moderate-to-severe persistent pain.[1] Many pain sufferers receive prolonged opioid administration. Unfortunately, however, tolerance develops, leading to the requirement for increasing opioid doses for adequate pain control.[1,2] Opioid tolerance is associated with the development of dependence and unpleasant withdrawal when treatment stops. Additional complications of opioid analgesics include constipation and, at higher doses, respiratory depression.[1] The requirement for escalating doses to maintain analgesia increases the potential for prescription opioid misuse, diversion, and overdose.[3,4] Despite intensive attempts to develop alternative analgesics, there are currently none to replace opioids in the treatment of severe pain. An alternative is to improve opioid analgesia, minimizing activation of pathways responsible for their detrimental effects, such as tolerance, either by seeking agonists biased against such pathways or by inhibiting them with adjunct agents.[5,6]

What We Already Know about This Topic

- Analgesic tolerance limits the clinical use of opioids for the management of chronic pain
- β-Arrestin2, a protein that recruits kinases such as c-Src to the μ opioid receptor, is critical for morphine analgesic tolerance

What This Article Tells Us That Is New

- The c-Src inhibitor dasatinib attenuated and reversed morphine-induced tolerance in mice
- Dasatinib did not alter the locomotor or use reinforcing effects of morphine in mice

μ-Opioid receptors mediate both the beneficial and the adverse effects of analgesic opioids.[7] μ Receptors are G protein–coupled receptors that also recruit β-arrestin2, which participates in desensitization, endocytosis, and signaling through various kinases, including extracellular signal-regulated kinase (ERK) and the nonreceptor tyrosine kinase c-Src.[2,8] Mice lacking β-arrestin2 (β-arr2$^{-/-}$ mice) exhibit reduced morphine tolerance and increased μ receptor–mediated basal nociception.[9,10] The inhibition of several pathways

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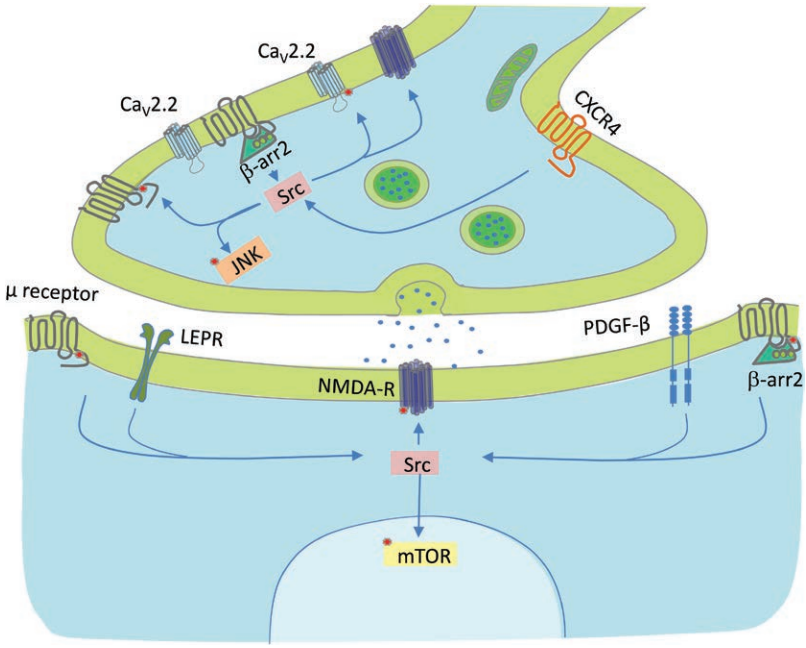

Fig. 1. Pathways implicated in tolerance that converge on Src. Neurons contain high levels of c-Src.[21] Recent studies have identified several pathways that converge on Src, and their inhibition reduces morphine tolerance,[6,38–42] potentially implicating the nonreceptor tyrosine kinase as a hub in this process. *Red spots* represent targets of Src-mediated phosphorylation.[24,25,43] μ Receptors (*gray*), the chemokine receptor type 4 (CXCR4), the leptin receptor (LEPR), *N*-methyl-D-aspartate receptors (NMDA-R), and platelet-derived growth factor receptor β (PDGF-β) are depicted in *gray*, *red*, *green*, *dark blue*, and *light blue*, respectively. β-Arrestin2 (β-arr2), Src kinase (Src), c-Jun *N*-terminal kinase (JNK), mechanistic target of rapamycin (mTOR), and N-type Ca^{2+} channels ($Ca_V2.2$) are depicted in *green*, *pink*, *orange*, *yellow*, and *light blue*, respectively.

that converge on c-Src also reduces morphine tolerance, implicating the tyrosine kinase as a potential hub for this process (fig. 1).

The μ receptor–mediated activation of c-Src in primary afferent neurons requires β-arrestin2, and inhibition of c-Src causes reductions in μ receptor endocytosis and opioid-induced desensitization.[11,12] These observations led us to hypothesize that c-Src contributes to morphine tolerance. Given the evidence for a role of β-arrestin2 in the locomotor and reinforcing effects of morphine, we further hypothesized that c-Src also participates in these behaviors.[13,14] Our findings suggest that c-Src inhibition suppresses tolerance without altering the locomotor or reinforcing effects of analgesic opioids.

Materials and Methods

Animals

In this study, we used $\mu^{+/-}$, $\mu^{-/-}$, and $\beta\text{-arr2}^{-/-}$ mice maintained on the C57BL/6J background in the Ninewells Hospital Medical Resource Unit (Dundee, United Kingdom) in accordance with the local ethics committee and United Kingdom Home Office regulations with an appropriate project license. They had access to food and water *ad libitum* with 12-h cycles of light and dark, and the temperature was maintained between 19° and 21°C. All of the experiments were performed in the light phase. Mice used in experiments were genotyped by Transnetyx (USA).

Behavioral Tests

Before each experiment, mice (aged 7 to 24 weeks, both sexes) were habituated. All of the experiments took place during the light phase. Drug doses were calculated using individual body weight, and maximum volume administered in a single injection was 200 μl.

Drug Administration

Morphine sulphate (Sigma-Aldrich, United Kingdom) was diluted in 0.9% NaCl in an aseptic environment and filtered using a 0.2-μm syringe filter before use. Morphine was administered subcutaneously. For experiments involving c-Src inhibition, dasatinib (Bristol Myers Squibb, USA), PP2 (Tocris, United Kingdom), and PP3 (Tocris) were reconstituted in dimethyl sulfoxide and Kolliphor EL (Sigma-Aldrich) and diluted in a 0.9% saline solution. Dasatinib (5 mg/kg), its vehicle, PP2 (5 mg/kg), and PP3 (5 mg/kg) were administered *via* the intraperitoneal route. Mice were randomly assigned to vehicle- or drug-treated groups while balancing the proportion of males and females. All of the samples were included for analysis with one exception: a mouse incorrectly assigned as $\mu^{-/-}$, which was omitted due to the initial genotyping error. During tail withdrawal assays, the individual measuring the latency was blinded to the condition of the animal. Conditioned place preference and locomotor data were collected by closed-circuit television, and footage was

analyzed automatically by AnyMaze software (Stoelting Europe, Ireland). Sample sizes were chosen based on our previous experience.[10]

Tail Withdrawal Assay

Morphine analgesia was assessed by measuring the latency for tail withdrawal from 48°C water 30 min after subcutaneous administration. Maximum exposure time to 48°C water was 15 s. We used an electronic thermostatic circulating water bath (Thermo Fisher, United Kingdom) to maintain water temperature within ± 0.1°C. Baseline tail withdrawal latencies were measured before the start of each experiment.

Conditioned Place Preference

We used a two-compartment model of conditioned place preference to investigate morphine reinforcement in mice. One chamber had a wall covering of black and white horizontal stripes and the other black and white vertical stripes. The compartments are contained within an operant box. These boxes are soundproofed and allow light levels to be controlled at approximately 70 lumens. The temperature was maintained between 21° and 23°C. Mice were habituated to the testing environment and allowed free access to both chambers before experiments. Mouse activity was recorded using a closed-circuit television camera, and parameters such as time spent in each chamber and distance traveled were acquired using AnyMaze software. During the 4-day conditioning period, all of the mice received subcutaneous injections of 0.9% saline (volume matched to that of the morphine injection) in either chamber. Four hours later the mice received a subcutaneous injection of morphine sulphate in the opposite chamber. After each injection they were confined to the corresponding chamber for 30 min. Between conditioning sessions mice were returned to their home cages. On day 5 mice were allowed free access to both chambers for 15 min. The time spent in each chamber was recorded using AnyMaze software.

Cell Culture and Western Blots

SW620 human colon cancer cells were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin (Invitrogen, USA) at 37°C and 5% CO_2.[15] Cell lysis was performed in radioimmunoprecipitation assay buffer (Thermo-Fisher). Proteins were separated using denaturing sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. c-Src and phosphorylated c-Src proteins were probed with rabbit anti-Src and anti-p-Src antibodies, respectively (both from New England Biolabs, United Kingdom). Mouse antiactin antibody (Abcam, United Kingdom) was used as a loading control. The primary antibodies were visualized with enhanced chemiluminescence reactions (ECL Prime, GE Life Sciences, United Kingdom) using the appropriate horseradish peroxidase–conjugated secondary antibodies.

Data Analysis

Tail withdrawal latencies were calculated as a percentage of maximal possible effect (MPE). The %MPE is calculated using the following equation in which the maximum exposure time (MET) is 15 s and the basal latency was the time for tail withdrawal from 48°C water in the absence of drug administration:

$$\%MPE = \frac{test\ latency - basal\ latency}{MET - basal\ latency} \times 100$$

Comparison of conditioned place preference was performed using preference scores, calculated by subtracting the time spent in the saline paired chamber from the time spent in the morphine paired chamber. Morphine dose–response relationships were fitted with a logistic equation to determine ED_{50} values, using GraphPad Prism software (USA).

Statistics

Nonparametric %MPE values for tolerance studies, which do not conform to the normal distribution, are expressed as median ± interquartile range (IQR). All of the other data are expressed as mean ± SD. Statistical comparisons of the development of tolerance (%MPE values) were analyzed using the Kruskall–Wallis test. Pairwise analyses within genotypes (*vs.* day 1) and between genotypes (on the same days) were compared using the Dunn multiple comparison correction. Other pairwise statistical comparisons of parametric data (*i.e.*, distance and time) were made using two-tailed *t* tests (paired or unpaired, as indicated). Three or more groups were compared using one-way or two-way ANOVA, as appropriate. Repeated-measures ANOVA was used when data were acquired over multiple days. *Post hoc* pairwise testing was performed using either the Dunnett test (one-way ANOVA) or the Bonferroni test (two-way ANOVA). *P* values of less than 0.05 were considered statistically significant. Statistical testing was performed using GraphPad Prism software.

Results

Src Inhibition Attenuates Morphine Analgesic Tolerance

The subcutaneous administration of morphine caused a dose-dependent analgesia, prolonging tail withdrawal by C57BL/6 mice from 48°C water (fig. 2A). Consistent with a previous report,[7] this effect depended on μ receptor expression, as evidenced by a lack of morphine (10 mg/kg) analgesia in $\mu^{-/-}$ mice (fig. 2A). Furthermore, morphine was less potent in $\mu^{+/-}$ mice, which lack 50% of the full complement of μ receptors,[7] without alteration of maximal efficacy (fig. 2A; table 1).

We examined the development of tolerance to repeated once-daily injections of morphine (10 mg/kg subcutaneously). Using this paradigm, the analgesic effect declined in wild-type (WT) mice over several days, reflecting the gradual development of morphine tolerance (fig. 2B). There was a significant reduction of the prolongation of tail withdrawal, with respect to that recorded on day 1, on days 9 and 10 of morphine administration ($P < 0.05$; Kruskall–Wallis test; *post hoc* Dunn correction). Tolerance involves reduced μ receptor reserve through desensitization and endocytosis.[2]

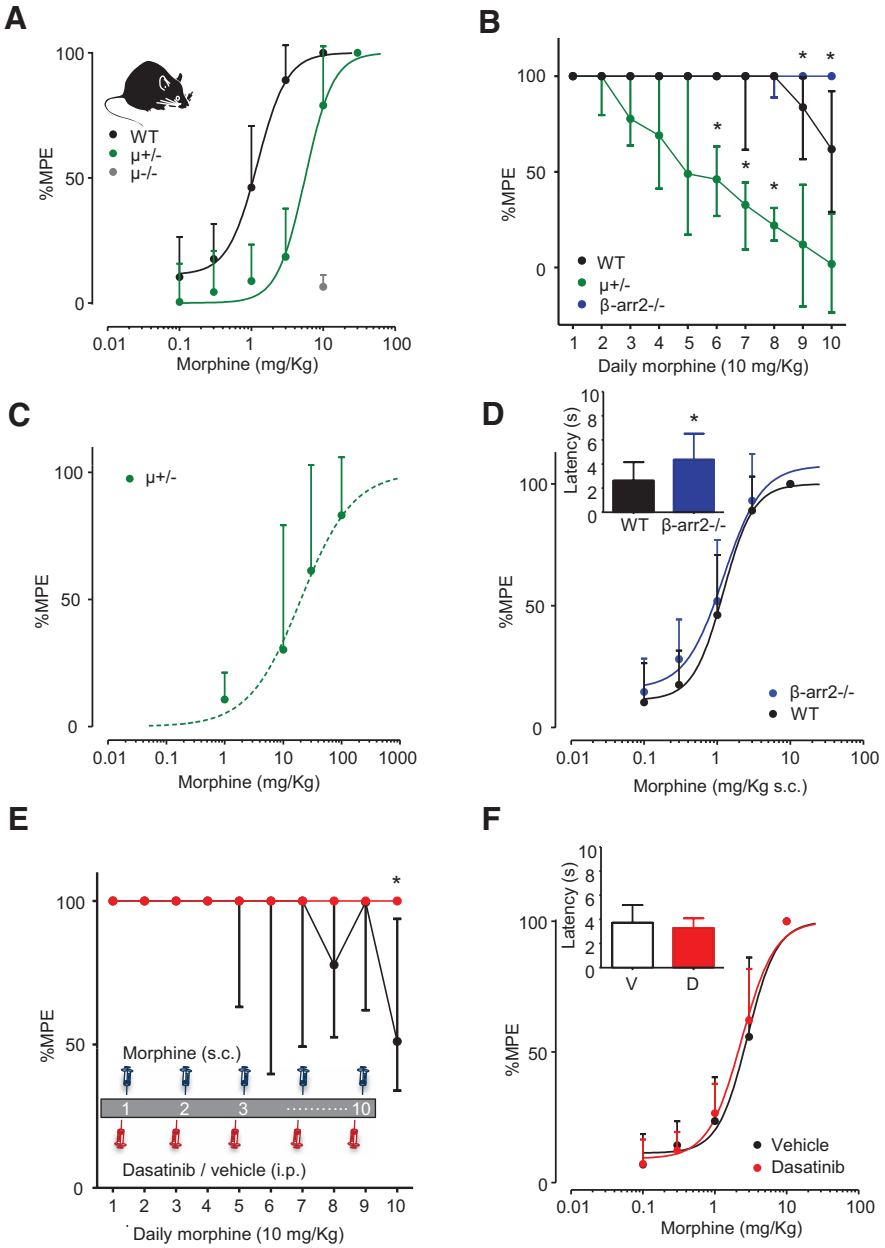

Fig. 2. Dasatinib attenuates morphine tolerance. (*A*) The dose dependence of morphine prolongation of latency for tail withdrawal from noxious heat in wild-type (n = 29) and $\mu^{+/-}$ mice (n = 15). The ED_{50} was significantly greater in $\mu^{+/-}$ mice (table 1). Morphine (10 mg/kg) had no effect on tail withdrawal latency when applied to $\mu^{-/-}$ mice (n = 15). (*B*) The development of morphine (10 mg/kg) analgesic tolerance in wild-type (n = 16), $\mu^{+/-}$ (n = 15), and β-arr2$^{-/-}$ (n = 15) mice. Data identified with *asterisks* were significantly different from equivalent wild-type data (*$P < 0.05$, Kruskall–Wallis test, *post hoc* Dunn correction). (*C*) The dose dependence of morphine prolongation of latency for tail withdrawal from noxious heat in $\mu^{+/-}$ mice, in which tolerance was induced by four once-daily injections of morphine (10 mg/kg subcutaneously; s.c.). The morphine dose–response relationship was examined on day 5 (n = 8). Compared with naive $\mu^{+/-}$ mice, tolerance caused a reduction in the analgesic potency of morphine (table 1). (*D*) The dose–response relationship for morphine in β-arr2$^{-/-}$ mice (n = 16) was similar to that of wild-type (WT) mice (table 1). *Inset*, tail withdrawal latency was longer for β-arr2$^{-/-}$ compared with WT mice (*$P < 0.001$, Student's *t* test). (*E*) Dasatinib (5 mg/kg intraperitoneally; i.p.), applied 30 min before morphine (10 mg/kg), reduced morphine tolerance in WT mice (n = 8) compared with vehicle-treated controls (n = 8). *Inset*, The *schematic* represents the dosing regimen. Data identified with *asterisks* were significantly different from equivalent vehicle data (*$P < 0.01$, Kruskall–Wallis test, *post hoc* Dunn correction). (*F*) Dasatinib affected neither the morphine dose–response relationship nor the time for tail withdrawal measured in the absence of morphine (*inset bar graph*). *Data points* in *B* and *E* represent median values, and *error bars* are ± interquartile range. All other data are expressed as mean ± SD.

Table 1. Influence of Genotype and Tolerance on Morphine Analgesia ED_{50}

Genotype	ED_{50} Morphine, mg/kg	n
WT	1.2 ± 0.1	29
$\mu^{+/-}$ opioid naive	5.9 ± 0.8*	15
$\mu^{+/-}$ opioid tolerant	38.0 ± 14.0†	8
β-arr2$^{-/-}$	1.5 ± 0.4	16

Opioid tolerance was established in $\mu^{+/-}$ mice by 4 days of morphine (10 mg/kg subcutaneously) injections.

*P < 0.05 (unpaired t test *vs.* WT); †P < 0.05 (unpaired t test *vs.* MOP$^{+/-}$ opioid-naive).

β-arr2 = β-arrestin2; μ = μ-opoid receptor; WT = wild-type.

Consistent with this, $\mu^{+/-}$ mice demonstrated a significant reduction by day 6 (P < 0.05; Kruskall–Wallis test; *post hoc* Dunn correction) in the morphine-evoked prolongation of tail withdrawal latency, with respect to that recorded on day 1. Pairwise comparisons of tail withdrawal latencies revealed that $\mu^{+/-}$ mice displayed significantly more tolerance than WT mice on days 6, 7, and 8 (P < 0.05; Kruskall–Wallis test; *post hoc* Dunn correction; fig. 2B). Examination of the morphine dose–response relationship in $\mu^{+/-}$ mice on day 4 revealed a significant (Student's t test, P < 0.05) reduction in analgesic potency compared with morphine-naive $\mu^{+/-}$ mice (fig. 2C; table 1).

The multifunctional anchoring protein, β-arrestin2, participates in opioid receptor endocytosis, and its absence leads to an up-regulation of μ receptors at the cell surface of primary afferent neurons.[12] Mice lacking β-arrestin2 (β-arr2$^{-/-}$ mice) also exhibit reduced morphine tolerance.[9] Consistent with these findings, β-arr2$^{-/-}$ mice, when treated once daily with morphine (10 mg/kg subcutaneously), exhibited less tolerance on days 9 and 10 than did WT mice (P < 0.05; Kruskall–Wallis test; *post hoc* Dunn correction; fig. 2B). β-Arr2$^{-/-}$ mice had an unaltered morphine dose–response relationship compared with WT mice (table 1) and increased basal latencies for tail withdrawal from 48°C water (Student's t test, P < 0.001; fig. 2D).

The activation of μ receptors in primary afferent neurons leads to a β-arrestin2–dependent stimulation of c-Src activation.[12] We tested the hypothesis that c-Src contributes to the development of morphine analgesic tolerance using the antileukemia c-Src inhibitor dasatinib, which crosses the blood–brain barrier in mice.[15] When administered once daily to WT mice, 30 min before morphine (10 mg/kg subcutaneously), dasatinib (5 mg/kg intraperitoneally) reduced the development of analgesic tolerance (fig. 2E). As observed previously (fig. 2B), morphine tolerance developed slowly in WT mice but was nevertheless diminished by dasatinib (fig. 2E). The attenuation of morphine tolerance was significant on day 10 (P < 0.01; Kruskall–Wallis test; *post hoc* Dunn correction; fig. 2E). On day 10, vehicle-treated mice exhibited morphine (10 mg/kg subcutaneously) analgesia that had declined to a median of 51% MPE (IQR, 34 to 94%; n = 8) of that on day 1. By contrast, dasatinib-treated mice maintained full analgesia on day 10. The attenuation of morphine analgesic tolerance by dasatinib occurred without alteration of either the morphine dose–response relationship or basal nociception (fig. 2F).

Due to the slow development of tolerance in WT mice, we examined the effects of dasatinib in $\mu^{+/-}$ mice in which tolerance develops faster (fig. 2B). Vehicle-treated $\mu^{+/-}$ mice developed morphine tolerance from day 4 (P < 0.05; Kruskall–Wallis test; *post hoc* Dunn correction; fig. 3A). Dasatinib reduced morphine tolerance in $\mu^{+/-}$ mice, and the attenuation was significant on day 5 compared with vehicle-treated $\mu^{+/-}$ mice (P < 0.05; Kruskall–Wallis test; *post hoc* Dunn correction; fig. 3A). By day 5 of daily morphine administration, analgesia in $\mu^{+/-}$ mice receiving vehicle intraperitoneally had declined to a median of 34% MPE (IQR, 5 to 52%; n = 8) of that seen on day 1. By contrast, on day 5, $\mu^{+/-}$ mice receiving dasatinib (5 mg/kg intraperitoneally) 30 min before morphine maintained a median of 100% MPE (IQR, 68 to 100%; n = 8) analgesia (fig. 3A).

Although c-Src inhibition is considered responsible for its clinical efficacy, dasatinib also inhibits other tyrosine kinases.[16] By comparison, PP2 is more specific and has the advantage of the inactive analog PP3, which can be used as a comparator.[12] We tested the inhibitory effects of dasatinib, PP2, and PP3 in SW620 colon cancer cells, which have high levels of basal c-Src activity.[17] Consistent with their reported properties, dasatinib and PP2 inhibited c-Src when administered to colon cancer cells, whereas PP3 was inactive (fig. 3B). We administered PP2 or PP3 (5 mg/kg intraperitoneally) to $\mu^{+/-}$ mice once daily 30 min before morphine (10 mg/kg subcutaneously). PP3 had no effect; the development of morphine tolerance was similar to that seen in vehicle-treated mice. However, PP2 attenuated tolerance from day 4 when compared with PP3-treated mice (P < 0.05; Kruskall-Wallis test; *post hoc* Dunn correction; fig. 3C). Morphine analgesia in $\mu^{+/-}$ mice declined to a median of 20% MPE (IQR, 14 to 25%; n = 12) of its level on day 1 in mice receiving PP3. By contrast, on day 5, $\mu^{+/-}$ mice receiving PP2 maintained 91% MPE (IQR, 78 to 100%; n = 12) of the analgesia seen on day 1. Neither dasatinib nor PP2 (or PP3) affected basal tail withdrawal when applied to $\mu^{+/-}$ mice in the absence of morphine (data not shown).

Src Inhibition Does Not Affect the Psychomotor Effects of Morphine

In addition to analgesia, morphine evokes psychomotor-stimulatory and reinforcing effects in mice.[13,14] Compared with saline injections (19 ± 4 m traveled on day 1, 30 min after injection), WT mice administered morphine (10 mg/kg subcutaneously) exhibited dramatically increased locomotor activity (81 ± 20 m, n = 8, traveled on day 1, 30 min after injection), quantified by analysis of video tracking (P < 0.05, paired t test; fig. 4A). By contrast, locomotor stimulation was absent from $\mu^{-/-}$ mice administered morphine (10 mg/kg), in which the average distances traveled were 19 ± 5 m and 17 ± 6

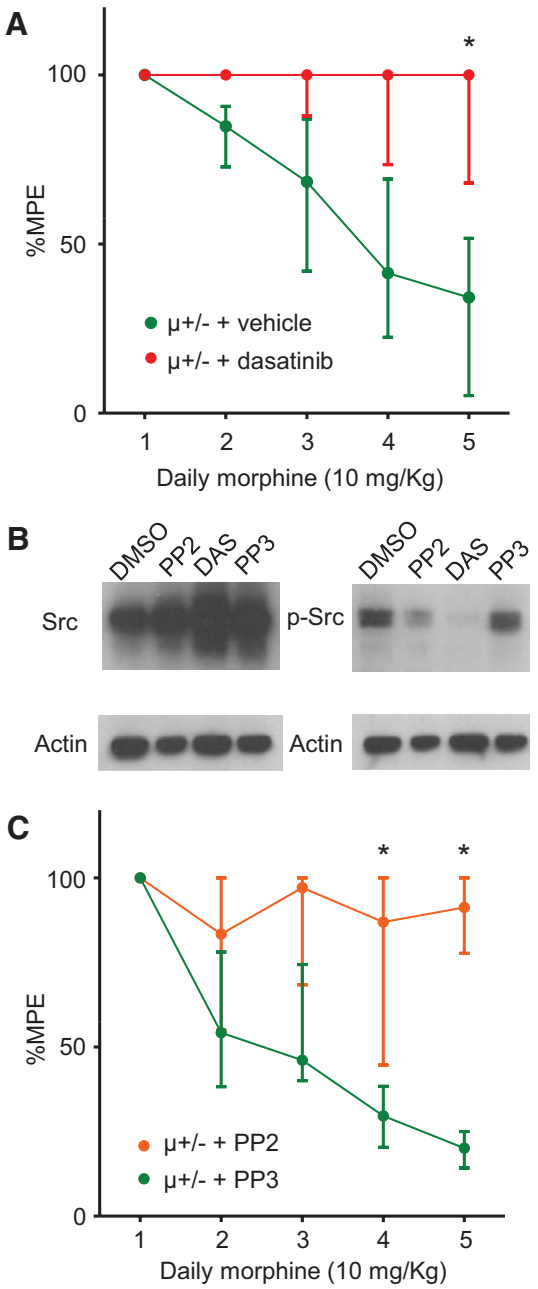

Fig. 3. Inhibition of c-Src attenuates morphine tolerance. (*A*) Dasatinib (n = 8) reduced tolerance in $\mu^{+/-}$ mice compared with vehicle injections (n = 8). Data identified with *asterisks* were significantly different from equivalent vehicle data (*$P < 0.05$, Kruskall–Wallis test, *post hoc* Dunn correction). (*B*) Western blot showing total c-Src (*left panel*) and phosphorylated c-Src (*right panel*) extracted from SW620 colon cancer cells treated with dimethyl sulfoxide (DMSO; vehicle), PP2 (10 μM), dasatinib (DAS; 10 μM), or PP3 (10 μM). β-Actin was used as a loading control. PP2 and dasatinib reduced phosphorylated c-Src levels, whereas PP3 had no effect relative to vehicle. (*C*) The relatively selective c-Src inhibitor PP2 (5 mg/kg intraperitoneally; i.p.) also attenuated the development of morphine tolerance, whereas the inactive analog PP3 (5 mg/kg i.p.) did not. Data identified with *asterisks* were significantly different from equivalent PP3 data (*$P < 0.01$, Kruskall–Wallis test, *post hoc* Dunn correction). Data are presented as median ± interquartile range.

m on day 1 after saline and morphine injections (n = 7), respectively (fig. 4A). Morphine (10 mg/kg) was without an effect on locomotion in $\mu^{-/-}$ mice on all 3 days of administration (fig. 4B). By contrast, morphine (3 mg/kg) caused a modest enhancement of locomotion in WT mice (fig. 4C). However, there was neither an effect of time *per se* nor a significant interaction of morphine and time (drug $F_{1,28} = 5.8$, $P < 0.05$, time $F_{2,28} = 0.98$, $P = 0.4$, interaction $F_{2,28} = 2.9$, $P = 0.07$; two-way ANOVA). By contrast, repeated daily morphine (10 mg/kg) administration caused sensitization of locomotor stimulation in WT mice (drug $F_{1,28} = 72$, time $F_{2,28} = 12$, interaction $F_{2,28} = 17$, all $P < 0.0005$; two-way ANOVA; fig. 4D).

By contrast to WT mice, morphine (10 mg/kg) locomotor stimulation was modest in $\mu^{+/-}$ mice and did not exhibit sensitization (drug $F_{1,28} = 36$, $P < 0.0001$, time $F_{2,28} = 1.7$, $P = 0.2$, interaction $F_{2,28} = 2.5$, $P = 0.1$; two-way ANOVA; fig. 4E). A higher dose of morphine (30 mg/kg) evoked a more robust locomotor stimulation accompanied by sensitization (drug $F_{1,28} = 45$, $P < 0.0001$, time $F_{2,28} = 3.1$, $P = 0.06$, interaction $F_{2,28} = 8.4$, $P < 0.001$; two-way ANOVA; fig. 4F).

The psychomotor effect of morphine is also influenced by β-arrestin2 expression.[13,14] In keeping with previous reports, β-arr2$^{-/-}$ mice displayed a diminished morphine (10 mg/kg subcutaneously) locomotor stimulatory response compared with WT mice (fig. 4A). Morphine increased ($P < 0.01$, paired *t* test) the average distance traveled by β-arr2$^{-/-}$ mice to 51 ± 19 m (n = 8) compared with 16 ± 5 m (n = 8) in β-arr2$^{-/-}$ mice receiving vehicle. When compared with the locomotor stimulation by morphine (10 mg/kg) exhibited by WT mice, the effect of morphine in β-arr2$^{-/-}$ mice was significantly ($P < 0.01$, Student's *t* test) diminished. Although there was no significant effect of the lower dose of morphine (3 mg/kg) on locomotion in β-arr2$^{-/-}$ mice (fig. 4G), mice receiving 10 mg/kg morphine exhibited increased locomotion and sensitization (drug $F_{1,28} = 31$, time $F_{2,28} = 6.7$, interaction $F_{2,28} = 21$, all $P < 0.005$; two-way ANOVA; fig. 4H).

The requirement for β-arrestin2 for the full locomotor stimulatory response to morphine (fig. 4A) has been linked to its role in recruiting phospho-ERK to D1 receptors in the striatum. Inhibition of mitogen-activated protein kinase/ERK kinase, which phosphorylates and thereby activates ERK, reduces morphine locomotor stimulation.[14] We used dasatinib to determine whether c-Src is also involved in the stimulation of locomotion by morphine. When administered daily (5 mg/kg intraperitoneally) either alone (n = 8) or 30 min before morphine (n = 8), dasatinib affected neither the average basal locomotion nor the average morphine locomotor stimulation compared with mice receiving vehicle (fig. 5A). Dasatinib had no effect on locomotion on any of the 3 days of its sole administration (fig. 5B). Furthermore, morphine (10 mg/kg) caused locomotor stimulation and sensitization when administered on days 1 to 3 either after vehicle (drug $F_{1,28} = 561$, time $F_{2,28} = 18$, interaction

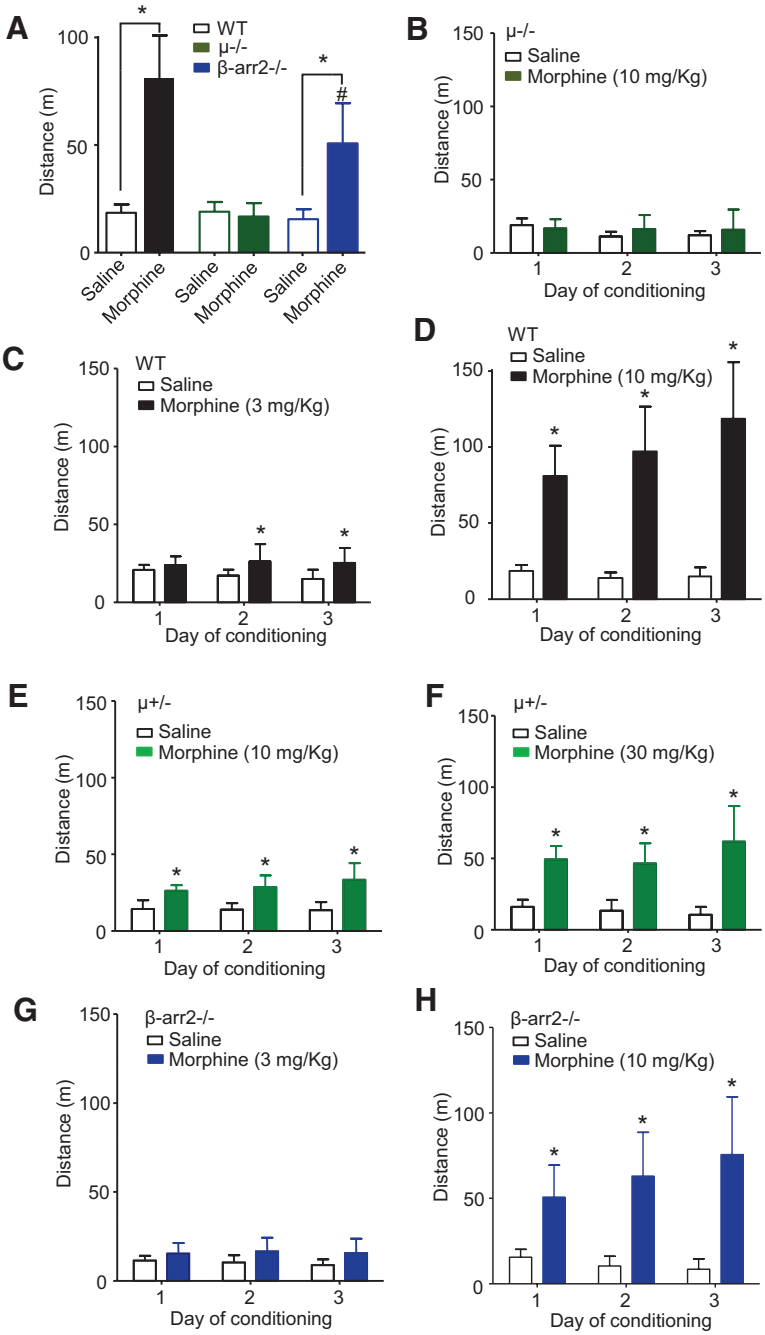

Fig. 4. Either fewer μ receptors or the absence of β-arrestin2 diminishes psychomotor stimulation by morphine. (*A*) Morphine (10 mg/kg subcutaneously) stimulated locomotor activity in wild-type (WT) mice averaged over the 3 days of conditioning (*P < 0.001, n = 8, paired t test compared with saline). This effect was not seen in $\mu^{-/-}$ mice in which morphine had no effect on the averaged locomotion (n = 7). By contrast, morphine stimulated locomotion in β-arr2$^{-/-}$ mice (*P < 0.01, paired t test; n = 8), but the average distance traveled was less than that of WT mice (#P < 0.01, unpaired t test). (*B*) Morphine (10 mg/kg) was without effect on distance traveled by $\mu^{-/-}$ mice (n = 7) on all days of conditioning. (*C*) Morphine (3 mg/kg) administration to WT mice (n = 8) showed a modest increase on distance traveled on days 2 and 3 of conditioning (*P < 0.05, two-way ANOVA, *post hoc* Bonferroni test). (*D*) At a higher dose (10 mg/kg subcutaneously), morphine increased distance traveled on all 3 days, and this effect exhibited sensitization (*P < 0.0001, two-way ANOVA, *post hoc* Bonferroni test; n = 8). (*E*) The locomotor effect of morphine (10 mg/kg) was diminished in $\mu^{+/-}$ mice (n = 8), and there was no sensitization (*P < 0.01 on day 1, P < 0.0001 on days 2 and 3, two-way ANOVA, *post hoc* Bonferroni test). (*F*) A higher dose of morphine (30 mg/kg) enhanced locomotion, but there was no effect of time over the 3 days of conditioning (*P < 0.0001, two-way ANOVA, *post hoc* Bonferroni test; n = 8). (*G*) Morphine (3 mg/kg) had no effect on distance traveled by β-arr2$^{-/-}$ mice on days 1 to 3 (n = 8). (*H*) At a higher dose (10 mg/kg), morphine increased distance traveled by β-arr2$^{-/-}$ mice on all 3 days, and this effect exhibited sensitization (*P < 0.01 on day 1, P < 0.0001 on days 2 and 3, two-way ANOVA, *post hoc* Bonferroni test; n = 8).

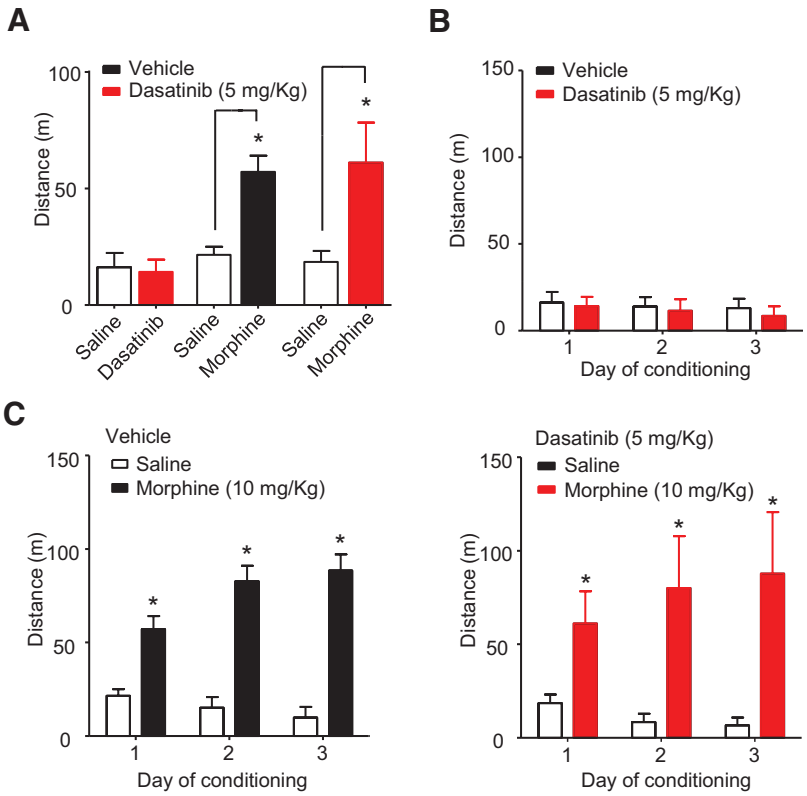

Fig. 5. Dasatinib does not affect psychomotor stimulation by morphine. (*A*) Graph of locomotion averaged over 3 days of conditioning with dasatinib and/or morphine. Dasatinib had no effect on locomotion when administered alone to wild-type mice (n = 8). Dasatinib (n = 8) also had no effect compared with vehicle (n = 8) on the stimulation of locomotion by morphine, which was significant in both cases (*$P < 0.001$, paired *t* test). (*B*) Dasatinib (5 mg/kg) was without effect on distance traveled by mice (n = 8) on all 3 days of conditioning. (*C*) Morphine caused locomotor stimulation with sensitization over the 3 days of conditioning when mice were administered either vehicle (*left panel*) or dasatinib (*right panel*) intraperitoneally (*$P < 0.0001$, two-way ANOVA, *post hoc* Bonferroni test).

$F_{2,28}$ = 72, all $P < 0.0001$; two-way ANOVA) or dasatinib (drug $F_{1,28} = 64$, $P < 0.0001$, time $F_{2,28} = 1.5$, $P = 0.24$, interaction $F_{2,28} = 11$, $P = 0.0003$; two-way ANOVA; fig. 5C).

Src Inhibition Does Not Affect Morphine Reinforcement

Conditioned place preference represents drug reinforcement, an important component of human substance misuse.[18] Although there was no preference on day 1 of conditioning, WT mice exhibited a clear preference for the environment that was paired with morphine (10 mg/kg subcutaneously) administration on day 5 after conditioning ($P < 0.01$, one-way ANOVA with *post hoc* Dunnett test *vs.* no morphine; fig. 6A). Increased time spent in the morphine (10 mg/kg) paired environment was evident throughout the 15-min testing period with no influence of time (drug $F_{1,42} = 143$, $P < 0.0001$, time $F_{2,42} < 0.0001$, $P = 1.0$, interaction $F_{2,42} = 0.2$, $P = 0.86$; two-way ANOVA; fig. 6B). In confirmation of the essential role for μ receptors in this reinforcing effect, $\mu^{-/-}$ mice lacked preference for the morphine (10 mg/kg) paired environment (fig. 6, A and C). $\mu^{-/-}$ mice spent equal times in the environments paired with either saline or morphine at all stages during testing (fig. 6C). Similarly, $\mu^{+/-}$ mice exhibited no preference for the morphine (10 mg/kg) paired environment (fig. 6D). Morphine preference did however become apparent throughout the 15-min period in $\mu^{+/-}$ mice receiving the higher dose of 30 mg/kg morphine (drug $F_{1,28} = 19$, $P < 0.0005$, time $F_{2,28} < 0.0001$, $P = 1.0$, interaction $F_{2,28} = 1.2$, $P = 0.32$; two-way ANOVA; fig. 6E). Comparison of the dose dependence of morphine preference in $\mu^{+/-}$ mice reveals an apparent dextral shift compared with wild-type mice (fig. 6F *vs.* fig. 6A), with morphine (30 mg/kg) causing a significant preference ($P < 0.01$, one-way ANOVA with *post hoc* Dunnett test *vs.* no morphine).

A previous study demonstrated that an absence of β-arrestin2 enhances the rewarding properties of morphine.[13] In agreement with this we found that β-arr2$^{-/-}$ mice exhibited an increased sensitivity to morphine-conditioned place preference (fig. 6G). Unlike WT mice that lacked a significant response to 3 mg/kg morphine, the same dose caused a robust conditioned place preference in β-arr2$^{-/-}$ mice ($P < 0.01$, one-way ANOVA with *post hoc* Dunnett test *vs.* no morphine), which was similar to that associated with 10 mg/kg morphine (fig. 6G). These findings suggest that inhibition of a β-arrestin2–mediated signaling pathway may increase reward. We next examined

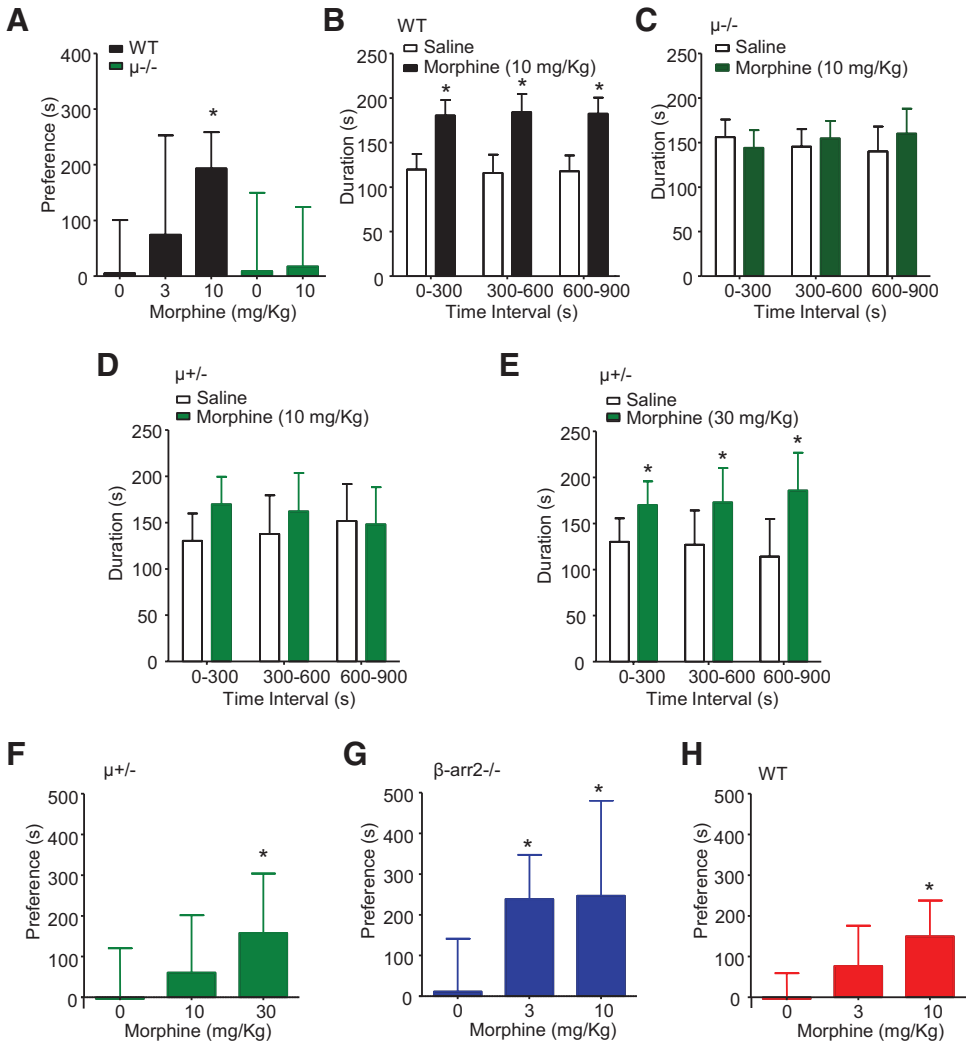

Fig. 6. Unlike the absence of β-arrestin2, which enhances reinforcement by morphine, dasatinib had no effect. (*A*) Morphine (3 and 10 mg/kg subcutaneously) caused a dose-dependent preference of wild-type (WT) mice (n = 8) for the paired environment (**P* < 0.01, one-way ANOVA, *post hoc* Dunnett test). Morphine preference was lacking in $\mu^{-/-}$ mice (n = 7). (*B*) After conditioning, the duration of occupancy of the morphine-paired environment increased significantly compared with the saline-paired environment in WT mice at all three 5-min intervals (**P* < 0.0001, two-way ANOVA, *post hoc* Bonferroni test; n = 8). (*C*) $\mu^{-/-}$ mice (n = 7) exhibited no morphine conditioned place preference at any stage during the 15-min test period. (*D*) $\mu^{+/-}$ mice (n = 8) also exhibited no morphine (10 mg/kg) conditioned place preference. (*E*) By contrast, $\mu^{+/-}$ mice (n = 8) exhibited conditioned place preference to a higher dose of morphine (30 mg/kg; **P* < 0.05 for 300 to 600 s, *P* < 0.001 for 600 to 900 s, two-way ANOVA, *post hoc* Bonferroni test). (*F*) Morphine (10 and 30 mg/kg) caused a dose-dependent preference of $\mu^{+/-}$ mice (n = 8) for the paired environment (**P* < 0.05, one-way ANOVA, *post hoc* Dunnett test). (*G*) Morphine preference occurred at a lower dose in β-arr2$^{-/-}$ mice (**P* < 0.01, one-way ANOVA, *post hoc* Dunnett test; n = 8). (*H*) By contrast, dasatinib had no effect on morphine preference (**P* < 0.01, one-way ANOVA, *post hoc* Dunnett test; n = 8).

whether dasatinib (5 mg/kg intraperitoneally) causes a similar increase in morphine-conditioned place preference. Comparison of the dose dependence for conditioned place preference reveals that, unlike the absence of β-arrestin2, which enhanced the sensitivity to morphine preference in β-arr2$^{-/-}$ mice, the dose dependence in dasatinib-treated WT mice resembles that seen in untreated WT mice, with morphine preference observed at the 10-mg/kg dose (*P* < 0.01, one-way ANOVA with *post hoc* Dunnett test *vs.* no morphine; fig. 6H).

Dasatinib Reverses Morphine Analgesic Tolerance

Having established that dasatinib inhibits morphine tolerance without affecting reward, we explored whether dasatinib influences tolerance in mice in which it had already developed. $\mu^{+/-}$ mice were given morphine (10 mg/kg) daily to initiate the development of tolerance and on day 4 received either dasatinib or vehicle 30 min before morphine administration (fig. 7). Tolerance continued to develop in vehicle-treated mice. However, dasatinib caused an immediate reversal of tolerance and attenuated its additional development. Comparisons of

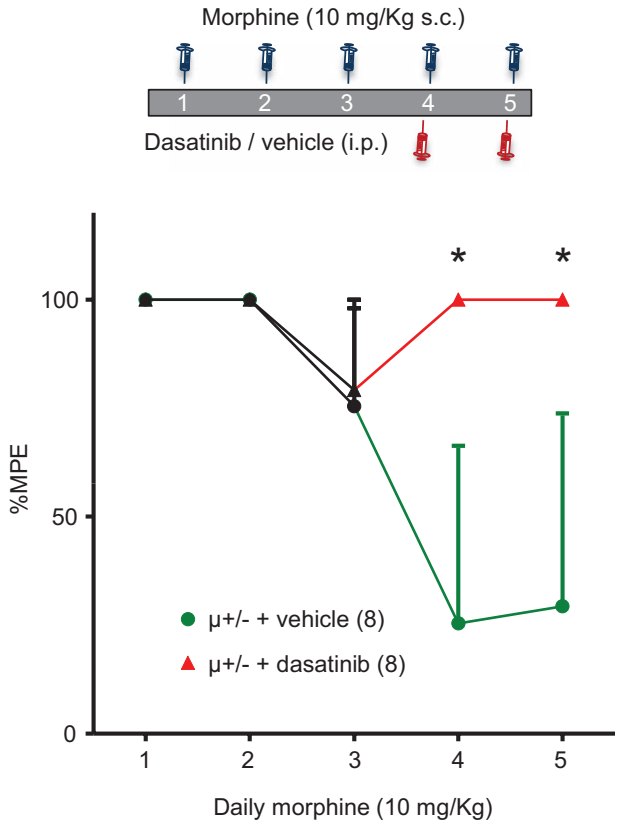

Fig. 7. Reversal of morphine tolerance by dasatinib. The diagram depicts the morphine, dasatinib–vehicle injection schedule on days 1 to 5. Data in the graph are average tail withdrawal latencies expressed as percentage of maximal possible effect (%MPE). $\mu^{+/-}$ mice injected with vehicle (n = 8) 30 min before morphine on days 4 and 5 continued to develop tolerance. By contrast, $\mu^{+/-}$ mice receiving dasatinib (n = 8) 30 min before morphine on days 4 and 5 exhibited reversal of analgesic tolerance. Data identified with *asterisks* were significantly different from equivalent vehicle data (*$P < 0.05$, Kruskall–Wallis test, *post hoc* Dunn correction). Data are presented as median ± interquartile range. i.p. = intraperitoneal; s.c. = subcutaneous.

analgesia on days 4 and 5 between vehicle- and dasatinib-treated mice revealed a statistically significant difference ($P < 0.05$; Kruskall–Wallis test; *post hoc* Dunn correction; fig. 7).

Discussion

This study reveals a requirement for c-Src activity for morphine analgesic tolerance and identifies c-Src inhibitors as agents that promote sustained analgesia. The c-Src inhibitor dasatinib not only attenuated tolerance but when administered before morphine also rapidly restored analgesia that had diminished during the preceding days. These effects occurred without altered psychomotor or reinforcing effects of morphine, suggesting that inhibitors of c-Src reduce opioid tolerance without increasing reward.

The c-Src inhibitors alone had no effect on nociception, and dasatinib did not influence the dose dependence of analgesia by morphine. These findings suggest that tolerance is required for c-Src inhibitors to enhance morphine analgesia. However, it remains to be determined whether c-Src activity is necessary for the expression and/or the development of tolerance. It is challenging to derive mechanistic insights from behavioral experiments. However, in the future it would be worthwhile to investigate whether the reversal of morphine analgesic tolerance persists after elimination of the c-Src inhibitor, as has been demonstrated in the case of an *N*-methyl-D-aspartate (NMDA) receptor antagonist.[19] Such an effect would be consistent with a requirement for c-Src activity for the development of tolerance.

The nonreceptor tyrosine kinase v-Src was the first retroviral oncogene to be discovered.[20] Subsequent research identified its cellular counterpart, c-Src, in vertebrates in which it is highly enriched at the synapse implying a role for the kinase in regulating neurotransmission.[21] G protein–coupled receptors, including μ receptors, couple to c-Src through mechanisms that are either independent (*e.g.*, a protein kinase C–mediated mechanism) or dependent on β-arrestins.[12,22–24] μ Receptor–mediated activation of c-Src in dorsal root ganglion neurons, which depends on β-arrestin2, contributes to inhibition of presynaptic voltage-activated Ca^{2+} channels through phosphorylation of a specific alternatively spliced isoform of the N-type channel.[12,25] In addition to its immediate role in μ receptor–mediated signal transduction, β-arrestin2–dependent c-Src activity also participates in μ receptor endocytosis and desensitization. The c-Src inhibitor PP2 increases surface expression of μ receptors in dorsal root ganglion neurons and decreases opioid-induced heterologous desensitization in locus ceruleus neurons.[11,12] These mechanisms may contribute to the attenuation of morphine analgesic tolerance by c-Src inhibitors *in vivo*.

Tolerance is arguably the most problematic aspect of opioid analgesia. The phenomenon leads to a requirement for escalating doses in patients suffering from persistent pain. Those on weak opioids often progress to stronger options, and the continuing proliferation of prescriptions for strong opioids has led to their increased availability for diversion and misuse.[1,4] The demonstration of a role for β-arrestin2 in tolerance and other side effects of opioids triggered the search for μ receptor agonists biased in favor of G protein stimulation.[5,26–31] The first was herkinorin, which activates G proteins without the recruitment of β-arrestin2 and produces analgesia in rats with markedly decreased tolerance compared with that of morphine.[28,29] Herkinorin also caused less respiratory depression and constipation, μ receptor–mediated side effects of morphine that are dependent on β-arrestin2 expression.[29,32] The discovery of herkinorin was followed by TRV130 and PZM21, additional analgesic μ receptor agonists biased against β-arrestin2 recruitment.[26,27] Although the relative tendency for these agonists to cause tolerance remains unreported, both cause negligible β-arrestin2 recruitment and less respiratory depression and constipation than are associated with morphine. TRV130

performed well as an acute pain medication during bunionectomy in a phase II clinical trial.[31] However, no biased μ receptor agonist has yet been tested in patients suffering from persistent pain. Furthermore, the extent that G protein bias plays in the apparently superior analgesic profiles of these new molecules compared with morphine remains unclear. An alternative explanation may be partial efficacy.[33]

An alternative to developing agonists biased against β-arrestin2 is to inhibit downstream components of the pathway, such as c-Src; our findings suggest that this is an effective approach for attenuating opioid tolerance. It is advantageous that c-Src inhibition, unlike deletion of β-arrestin2, does not influence the reinforcing or psychomotor effects of morphine. This suggests that c-Src inhibitors are unlikely to increase the hedonic effects of opioids. Although ERK has been implicated in mediating the influence of β-arrestin2 on psychomotor activation,[14] the cause of the enhanced sensitivity to morphine reinforcement in β-arr2$^{-/-}$ mice remains unknown. It is possible that this reflects an upregulation of surface μ receptors and/or dopamine receptors in the reward pathway in the absence of β-arrestin2–dependent endocytosis. If so, our findings suggest that this does not involve c-Src. Additional work is required to establish whether c-Src participates in other side effects of morphine, such as constipation and respiratory depression.

Although c-Src inhibitors were not antinociceptive in the acute pain model used in our study, c-Src activity has been implicated in persistent inflammatory, neuropathic, and bone cancer pain, in which c-Src inhibitors reduce hyperalgesia.[34–37] Hyperalgesia is associated with Src-mediated phosphorylation of the NMDA receptor, which leads to enhanced excitatory transmission in spinal neurons.[34,36] Several parallels can be drawn between hyperalgesia and morphine tolerance, including a common requirement for NMDA receptor activity[38]; the involvement of c-Src in both processes provides a potentially unifying mechanism. The ability of c-Src inhibitors to inhibit hyperalgesia and reverse tolerance, thereby restoring analgesia, makes them promising candidates as adjuncts to opioid analgesics.

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Competing Interests

The authors declare no competing interests.

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