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Early-life adversity increases morphine tolerance and persistent inflammatory hypersensitivity through upregulation of δ opioid receptors in mice

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Abstract

Exposure to severely stressful events during childhood is associated with poor health outcomes in later life, including chronic pain and substance use disorder. However, the mediators and mechanisms are unclear. We investigated the impact of a well-characterized mouse model of early-life adversity, fragmented maternal care (FC) between postnatal day 2 and 9, on nociception, inflammatory hypersensitivity, and responses to morphine. Male and female mice exposed to FC exhibited prolonged basal thermal withdrawal latencies and decreased mechanical sensitivity. In addition, morphine had reduced potency in mice exposed to FC and their development of tolerance to morphine was accelerated. Quantitative PCR analysis in several brain regions and the spinal cords of juvenile and adult mice revealed an impact of FC on the expression of genes encoding opioid peptide precursors and their receptors. These changes included enhanced abundance of δ opioid receptor transcript in the spinal cord. Acute inflammatory hypersensitivity (induced by hind paw administration of complete Freund's adjuvant) was unaffected by exposure to FC. However, after an initial recovery of mechanical hypersensitivity, there was a reappearance in mice exposed to FC by day 15, which was not seen in control mice. Changes in nociception, morphine responses, and hypersensitivity associated with FC were apparent in males and females but were absent from mice lacking δ receptors or β-arrestin2. These findings suggest that exposure to early-life adversity in mice enhances δ receptor expression leading to decreased basal sensitivity to noxious stimuli coupled with accelerated morphine tolerance and enhanced vulnerability to persistent inflammatory hypersensitivity.

Keywords: Pain, Hyperalgesia, Analgesia, Opiate, Neglect

1. Introduction

Repeated exposure to stressful early-life events, including neglect, abuse, and household dysfunction, increases the likelihood of poor health outcomes in adulthood.[26] There is a strong association between such adverse childhood experiences (ACEs) and problematic drug use, including opioid misuse.[11] Increased exposure to ACEs is also associated with chronic pain.[10,23,32,34,44] Chronic pain is a major global health burden affecting 20% to 50% of the population.[38] Unfortunately, options for its treatment are limited by the detrimental effects of analgesic drugs, particularly opioids.[5,6]

Opioid analgesics, including morphine, are recommended for the treatment of short-term moderate and severe pain. However, opioids provide little benefit for people with long-lasting pain. Morphine becomes less potent when administered repeatedly, through tolerance, which can lead to morphine induced hypersensitivity.[5,6,35] Opioid analgesics are also habit-forming in some people, potentially leading to addiction. Unfortunately, despite efforts to develop better drugs to treat severe pain, there are none as effective as opioids and, even with their limited ability to reduce persistent pain and their troubling side effects, opioids are prescribed to an increasing number of patients suffering from chronic pain.[5,22] People living in the most deprived communities receive the highest levels of opioid prescribing. Children living in deprivation also have a higher likelihood of exposure to ACEs,[21] which may contribute to an increased probability of poor health in later life.[15]

Several preclinical studies in rats demonstrate that exposure to neonatal adversity affects pain and responses to opioids assessed later in life.[4,19,20,24,27,33] However, few studies have examined the impact of early-life adversity on pain and opioid responses in mice, a species more amenable to the development of transgenic models for the exploration of potential underlying mechanisms. A recent study demonstrated that mice exposed to maternal separation exhibit a reduction in morphine antinociception associated with decreased μ and δ receptor mRNA expression within brainstem nuclei.[25]

Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

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In this study, we used the fragmented maternal care paradigm caused by limited bedding (FC), in male and female wild-type (WT), δ opioid receptor knockout (δ−/−), and β-arrestin2 knockout (β-arrestin2−/−) C57BL/6 mice. We tested the hypothesis that FC affects opioid tolerance, nociception, and inflammatory hypersensitivity through altered expression of opioid related genes in male and female mice. We examined the impact of FC on nociception, antinociceptive responses to the prototypical analgesic opioid, morphine, and persistent mechanical inflammatory hypersensitivity induced by complete Freund's adjuvant (CFA). We also identified transcripts encoding components of the opioid pathway that were differentially expressed in WT mice exposed to FC relative to control mice.

2. Methods

2.1. Animals

Male and female WT and transgenic C57Bl/6J mice lacking expression of δ receptors (δ−/−) or β-arrestin2 (β-arrestin2−/−) were used throughout this study.[35] Mice were used throughout their life span and varied in ages. Pups were reared with their dam in control or limited bedding cages from postnatal day (PD) 2 to 9. Gene expression was assessed in these mice as juveniles (PD 11-18) or adults (PD 60). Behavioural assessments were only performed in these mice as adults (PD 60) and occurred in a separate experimental room. All animals were maintained on a dimmable automated 12-hour light/dark cycle (8 AM-8 PM) at constant temperature (19-24°C) and provided with ad libitum access to food and water. Behavioural experiments were performed in the light phase in accordance with Home Office guidelines. All procedures were also approved by the local ethics board and ethical review board within the University of Dundee.

2.2. Fragmented care paradigm

Fragmented maternal care was established in mice using a well-characterised model involving exposure to limited nesting material during PD 2 to 9 as we and others previously described.[12,31] In brief, pregnant dams housed without a male were monitored for the birth of their litters every 12 hours and the day of birth designated PD 0. On PD 2, litter sizes were adjusted to <8 before dams (and their pups) were transferred to either control or FC cages assigned randomly before birth. Dams in control cages were provided with routine husbandry nesting material including a full-square (5-cm × 5-cm) nestlet of shredding material and ~650 mL of sawdust on the cage floor. By contrast, dams in FC cages were provided with 2/3 (by weight) of shredding material and ~60 mL of sawdust on the cage floor. The latter mice were additionally placed on a raised (2.5-cm) fine-gauge (50-mm) steel mesh platform preventing access to the sawdust beneath (Supplementary Figure 1A, available at http://links.lww.com/PAIN/B818). All dams and their pups were left undisturbed in these cages from PD 2 to 9 and video-recorded (iN-CAR CAM, Nextbase) daily at 08:00, 15:00, and 20:00 for 30 minutes each. On the morning of PD 9, all mice were transferred to clean cages containing routine husbandry materials.

2.3. Assessment of maternal behaviours

Dam–pup interactions and the number of nest departures made by the dam (termed sorties) were evaluated for all video recordings taken each day between PD 3 to 8 during the FC paradigm. Dam–pup interactions were scored every other minute throughout each recording (resulting in 15 × 1-minute epochs) by measuring the time (in seconds) that the dam was inside the nest and in full contact with her pups. Dam–pup interaction times were converted to a percentage of total time that each dam spent in contact with their pups on each day. The number of nest departures made by the dam (sorties), defined as all 4 paws outside of the nest, was scored continuously throughout each 30-minute recording. The number of sorties made during the paradigm was converted to sorties per hour for statistical comparisons. In addition, the impact of FC was monitored using the weights of each mouse. Mice were individually weighed before entering the early life paradigm (on PD 2), immediately after being removed (on PD 9), at weaning age (on PD 22), and as adults (on PD 60).

2.4. Quantitative polymerase chain reaction

Juvenile (PD 11-18) and adult (PD 60) mice were culled via cervical dislocation, and brain regions (prefrontal cortex, hippocampus, hypothalamus, thalamus, and the midbrain) or spinal cords were dissected and snap-frozen in liquid nitrogen. Because of the poor distinction between the thalamus and hypothalamus, both regions were combined into a thalamic-axis region in juvenile mice only.

Frozen tissue was thawed on ice submerged in 500 μL of TRIzol reagent (Thermo Fisher, Loughborough, United Kingdom) for 30 minutes and tissue mechanically homogenised using a tissue tearer (Cole Parmer, Cambridgeshire, United Kingdom) followed by sonication, on ice, for 30 seconds. RNA was precipitated using chloroform (100 μL per sample) and left to incubate at room temperature for 15 minutes. Samples were centrifuged at 12,000 rpm for 15 minutes at 4°C. The RNA containing aqueous phase was collected into fresh tubes and incubated with an additional 500-μL isopropanol at room temperature for 10 minutes. Samples were centrifuged again at 12,000 rpm for 10 minutes at 4°C and the supernatant discarded. Precipitated RNA was washed with 75% ethanol and centrifuged twice. Ethanol was carefully removed, and the pellet left to dry. Precipitated RNA was then resuspended in 30-μL nuclease-free water (Thermo Fisher) and stored at −80°C until required.

RNA was reverse-transcribed into cDNA using Superscript II (Invitrogen, Thermo Fisher) as per recommended instructions. Total RNA (1 μg) was diluted to a reaction volume of 13 μL in nuclease-free water containing 3-μg random primers (Invitrogen) and 1-μL undiluted (10-mM) deoxyribonucleotide triphosphate (Invitrogen). Samples were heated to 65°C for 5 minutes followed by brief incubation on ice. Reaction volumes were adjusted to 20 μL by adding 4-μL first strand buffer, 2-μL dichlorodiphenyltrichloroethane (0.1 M), and 1-μL SuperScript II reverse transcriptase (200 U/μL). Samples were incubated at room temperature for 2 minutes followed by incubation at 42°C for 50 minutes and a third incubation at 70°C for 15 minutes. Resulting cDNAs were diluted to a final concentration of 12.5 ng for quantitative PCR using nuclease-free water.

Primers (predesigned Life Technologies TaqMan gene expression array; Thermo Fisher) used for quantitative PCR were Oprm1 (μ opioid receptor; Mm01188089_m1), Oprd1 (δ opioid receptor; Mm01180757_m1), Oprk1 (κ opioid receptor; Mm01230885_m1), Pomc (proopiomelanocortin; Mm00435874_m1), Penk (proenkephalin; Mm01212875_m1), Pdyn (prodynorphin; Mm00457573_m1), Arrb2 (β-arrestin2; Mm00520666_g1), and Gapdh (glyceraldehyde 3-phosphate dehydrogenase; Mm99999915_g1). All primers used a 5′ FAM reporter dye and 3′ nonfluorescent quencher.

Quantitative PCR was performed in a 96-well assay format on a QuantStudio 7 Flex system (Applied Biosystems, Thermo Fisher)

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using a total reaction volume of 20 μL, which comprised the TaqMan gene expression array primer of interest (1 μL), diluted sample cDNA (2.5 μL), TaqMan array universal PCR mastermix II (10 μL), and nuclease-free water (6.5 μL). Amplification involved the 2-stage cycle threshold (CT) setup. Samples were initially held at 50˚C for 2 minutes then heated to 95˚C and held for a further 10 minutes. After, the plate was cycled between 60˚C (holding for 1 minute) and 95˚C (holding for 15 seconds) at a rate of 1.6˚C/seconds. Data acquired after 40 cycles were analysed using the comparative CT method ($2^{-\Delta\Delta CT}$) using *GAPDH* as the reference.

2.5. Tail withdrawal assay

The impact of FC on thermal nociception was measured using a modified version of the hot-water tail withdrawal assay as previously described.[3,36] Mice were restrained in polycarbonate tubes present in home cages and the distal third of their tails submerged into hot (48˚C) water maintained within 0.1˚C using a circulating water bath (thermostatic circulator bath Optima general purpose 12 L stainless steel tank, Fisher Scientific). The latency to tail withdrawal response (in seconds) was measured up to a maximum of 15 seconds to avoid tissue injury.

2.6. Morphine antinociception and tolerance

Dose–response relationships of morphine antinociception were constructed using the tail withdrawal assay as described above. Tail withdrawal latencies (TWLs) were measured at baseline followed by subcutaneous injection (s.c.) with morphine sulfate (Sigma, Merck Life Science UK Ltd, Gillingham, United Kingdom) into the scruff of the neck. All mice were administered with cumulative (0.1-100 mg/kg) doses of morphine and TWLs reassessed 30 minutes after injection with each morphine dose. The following day (day 2), and for 7 consecutive days thereafter (up to day 9), a single dose of morphine (10 mg/kg) was administered once daily to all mice and TWLs assessed immediately before and 30 minutes after injection to monitor the development of antinociceptive tolerance. On day 10, a second dose–response relationship was constructed using the same approach described above. Morphine was reconstituted daily to 2 mg/mL in sterile 0.9% saline and filtered using a 0.2-μm syringe filter. The maximum daily injection volume was limited to 0.1 mL/kg and dosed according to individual body weights.

2.7. Complete Freund's adjuvant model of persistent inflammation

Mice were restrained with a scruff and received a 10-μL intraplantar injection with undiluted (1 mg/mL) complete Freund's adjuvant (CFA; Merck Life Science UK Ltd). Complete Freund's adjuvant was administered into one hind paw at roughly the midline using a 29 G needle and 0.1-mL syringe. Neither anaesthetics nor analgesics were used for hind paw injections.

2.8. Mechanical sensitivity assay

The impact of FC on mechanical nociception was measured using an electronic von Frey filament (dynamic plantar aesthesiometer #37400-001, Ugo-Basile, Gemonio, Italy). Mice were individually housed in clear Perspex chambers on a raised mesh platform and habituated to the recording chamber for 30 minutes. A single filament delivering an increasing force at a linear rate of 2.5 g/s was directed at each hind paw from underneath the platform until a withdrawal response was elicited. Mechanical sensitivities were assessed once per day in the absence and presence of CFA-evoked inflammation, the latter in intervals of up to 5 days for a total of 30 days after injection with CFA. The threshold recorded for each mouse on each day represents the average of 3 replicates measured one after the other.

2.9. Data analysis

Tail withdrawal latencies (in seconds) for morphine antinociception are converted to a percentage of maximum possible effect (% MPE) using the calculation $100 \times$ (baseline latency $-$ morphine latency)/(15 $-$ baseline latency). The development of morphine antinociceptive tolerance is shown as the %MPE on each day during the 10-consecutive-day exposure, and, as the change in morphine ED_{50} (in mg/kg) on day 10 vs day 1 (ΔED_{50}) derived from morphine dose–response relationships of each mouse on day 1 and day 10, respectively. The expression of each transcript from quantitative PCR experiments is expressed relative to GAPDH from the same region of interest in the same mouse using the $2^{-\Delta\Delta CT}$ method. Raw data were processed using Microsoft Excel and imported into GraphPad Prism (version 5) or IBM SPSS Statistics (version 28.0.1.1) for visualisation and statistical analysis.

2.10. Statistics and data handling

Data analysis and interpretations conform to the recommendations set out by the ARRIVE guidelines and the National Institutes of Health.[28] Where sex differences were observed, the data are split and analysed according to sex. In cases where no apparent influence of sex was observed ($P > 0.05$), the data are pooled between sexes and analysed together. Data are represented as mean ± SD (quantitative PCR) or mean ± SEM (thermal nociception, mechanical nociception, morphine dose–response relationships, morphine tolerance, and CFA mechanical hypersensitivity). Data in graphical format are the averaged data derived from individual mice and shown for illustrative purposes. The number of animals used in each experiment is reported in the accompanying figure legends. Where appropriate, summary data are provided in the corresponding Supplemental Tables with statistics reported in the relevant legends (available at http://links.lww.com/PAIN/B818). Pairwise comparisons with *post hoc* corrections (where appropriate) were only performed on data sets detecting a statistically significant difference set at $P < 0.05$.

3. Results

3.1. Limited bedding caused fragmented care of neonatal mice

Consistent with our previous report and those of others,[12,31] exposure of C57BL/6 dams and their pups for 1 week to limited bedding (see Methods) during the postnatal period, PD 2 to PD 9, caused fragmented maternal care as defined by a significant increase in the number of sorties from the nest made by the dam (**Fig. 1A**). This behaviour occurred without altering the total dam–pup interaction time (**Fig. 1B**). Exposure to the limited bedding paradigm led to a reduction of body weight that recovered by PD 60 (Supplementary Figure 1A and B, available at http://links.lww.com/PAIN/B818). The impact of limited bedding on body weights up to PD 60 was similar for male and female offspring.

3.2. Fragmented care reduced acute thermal and mechanical nociception in wild-type mice

We assessed the impact of FC on acute thermal nociception using the warm-water tail withdrawal assay. Analysis of basal tail

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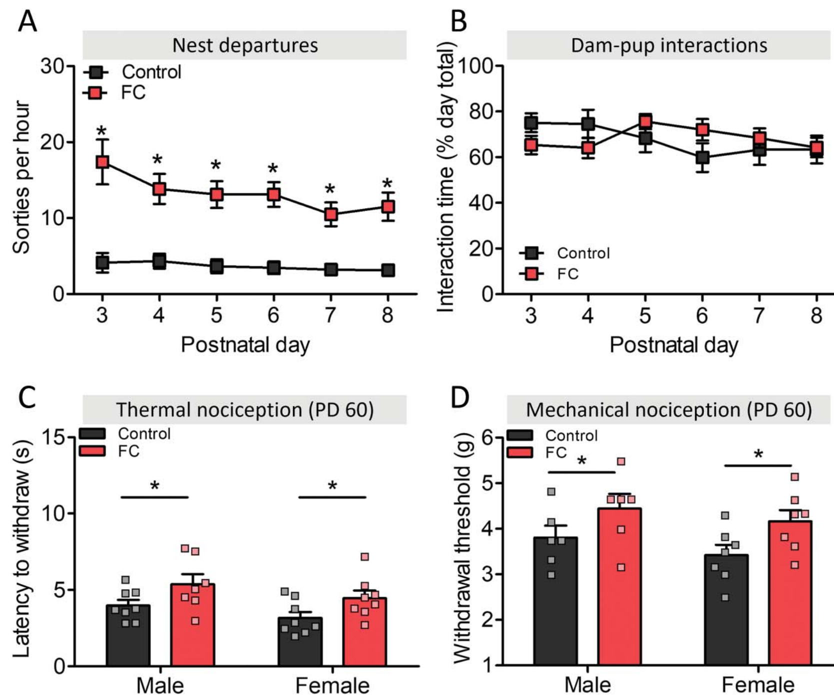

Figure 1. Fragmented care reduces acute thermal and mechanical nociception in wild-type male and female C57BL/6 mice. (A) Dams housed in fragmented care (FC) cages made significantly more nest departures compared with dams housed in control cages on postnatal day (PD) 3 to 8 (cage condition: $F_{1,100} = 52.5$, $P < 0.0001$; time $F_{5,100} = 1.7$, $P = 0.15$; interaction: $F_{5,100} = 0.89$, $P = 0.49$). (B) The time each dam spent interacting with their pups (expressed as a percentage of the total interaction time on each day) was similar between control and FC cages (cage condition: $F_{1,100} = 0.05$, $P = 0.82$; time $F_{5,100} = 0.88$, $P = 0.5$; interaction: $F_{5,100} = 1.9$, $P = 0.1$). (C) Mice exposed to FC during PD 2 to 9 had significantly prolonged tail withdrawal latencies from warm water (48°C) at PD 60 (cage condition: $F_{1,27} = 8.1$, $P = 0.01$; sex: $F = 3.3$, $P = 0.08$; interaction: $F_{1,27} = 0.01$, $P = 0.93$). (D) A separate cohort of mice exposed to FC during PD 2 to 9 had significantly enhanced mechanical thresholds on PD 60 (cage condition: $F_{1,22} = 6.9$, $P = 0.02$; sex: $F = 1.6$, $P = 0.22$; interaction: $F = 0.04$, $P = 0.85$). Statistical comparisons were performed using a 2-way ANOVA with Bonferroni correction. Data shown as mean ± SEM (bars) in all cases. Individual responses from each mouse are shown as separate datapoints. (A and B) n = 10 (control) and n = 12 (FC) separate cages. (C and D) n (control, then FC) = 14 and 13 (WT male), n = 15 and 15 (WT female). * $P < 0.05$ compared with control. ANOVA, analysis of variance; WT, wild-type.

withdrawal latencies in WT mice revealed that FC significantly prolonged the latencies of male and female mice compared with controls (**Fig. 1C**). Tail withdrawal latencies (mean ± SEM) in control mice were 4.0 ± 0.4 seconds (males) and 3.3 ± 0.4 seconds (females), while tail withdrawal latencies in FC mice were 5.4 ± 0.7 seconds (males) and 4.1 ± 0.3 seconds (females). These data demonstrate that FC reduces acute thermal nociception in both sexes.

We also examined acute mechanical sensitivities in control and FC mice using an automated von Frey (see Methods). Acute mechanical nociception was reduced in mice exposed to FC compared with controls and this occurred in both sexes (**Fig. 1D**). The withdrawal thresholds (mean ± SEM) in control mice were 3.9 ± 0.2 g (males) and 3.4 ± 0.1 g (females), while the withdrawal thresholds of mice exposed to FC were 4.4 ± 0.2 g (males) and 4.2 ± 0.2 g (females). These data demonstrate that FC reduces acute mechanical nociception in both sexes. In all subsequent analyses, female and male data are combined.

3.3. Fragmented care reduces the apparent potency of morphine and increases tolerance in wild-type mice

We examined the impact of FC on opioid-evoked antinociception by administering morphine at increasing doses (0.3, 1, 3, and 10 mg/kg s.c.) to adult mice (PD ≥ 60). Morphine dose-dependently prolonged tail withdrawal latencies reaching 100% of the maximal possible effect (MPE) after administration of 10 mg/kg to either control or FC mice (**Fig. 2A**). Dose–response data from each mouse were fitted using a logistic function. When averaged, the fitting parameters provided mean (with SEM) ED_{50} values for morphine of 2.3 ± 0.3 and 3.4 ± 0.3 mg/kg for control and FC mice, respectively. Comparison of the logED_{50} values (*t* test) revealed that prior exposure to FC caused a significant reduction in the apparent potency of morphine on day 1 (**Table 1**).

Repeated daily exposure causes tolerance to morphine (10 mg/kg) antinociception leading to a progressive diminution of the prolongation of tail withdrawal latency and a rightward shift in the dose–response relationship in C57BL/6 mice.[3] We examined the impact on morphine tolerance of exposure to FC. Mice exposed to FC exhibited an earlier onset of morphine tolerance as evidenced by a significant reduction (Friedman test) in antinociception on day 5 onward compared with day 1 of daily morphine (10 mg/kg). By contrast, control mice exhibited a significant reduction in MPE on day 8 through day 10 (**Fig. 2B**). Examination of morphine dose–response relationships on day 10 (**Fig. 2C**) revealed higher mean morphine ED_{50} values for mice exposed to FC (16.9 ± 1.6 mg/kg) compared with control mice (11.0 ± 0.6 mg/kg). Comparison of the logED_{50} values (*t* test) between WT control and FC mice on day 10 revealed that exposure to FC caused a significant reduction in the apparent potency of morphine (**Table 1**). A further comparison of ΔED_{50} values derived from morphine dose–response relationships on day 1 and day 10 revealed a greater shift in the apparent potency of morphine on day 10 in mice exposed to FC compared with controls (**Table 1**).

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Table 1

Morphine antinociception and the development of tolerance in wild-type, δ−/−, and β-arrestin2−/− control and fragmented care mice.

Genotype	Condition	Morphine		
		ED_{50} (mg/kg) Day 1	ED_{50} (mg/kg) Day 10	ΔED_{50}
Wild-type	Control	2.3 ± 0.3	11.0 ± 0.6	8.7 ± 0.6
	FC	3.4 ± 0.3*	16.9 ± 1.6*	13.5 ± 1.6*
δ−/−	Control	2.2 ± 0.3	4.1 ± 0.5	2.0 ± 0.6†
	FC	1.7 ± 0.4	3.6 ± 0.4	1.8 ± 0.7†
β-arrestin2−/−	Control	1.7 ± 0.3	5.7 ± 0.6	4.2 ± 0.7†
	FC	1.2 ± 0.2	5.0 ± 1.1	3.9 ± 1.3†

Dose–response relationships of morphine antinociception in WT (Fig. 2), δ−/−, and β-arrestin2−/− control and FC mice (Fig. 4) were fitted using a logistics function to determine morphine apparent potency (ED_{50}). Data derived from individual fits were log-transformed to compare potency between cage conditions and genotypes. Morphine's ED_{50} was reduced in WT FC mice relative to WT controls on day 1, and after 10 days of morphine (10 mg/kg) administration. In addition, FC caused a larger shift in apparent ED_{50} values in WT mice relative to controls. There was no difference in morphine ED_{50} on either day 1 or day 10, nor was there a significant difference in morphine ΔED_{50} values, between control or FC δ−/− or β-arrestin2−/− mice. However, morphine retained greater potency despite 10 days of injection in both δ−/− and β-arrestin2−/− mice. Data represent mean ± SEM. n (control, then FC) = 16, 15 (WT), n = 8, 11 (δ−/−), and n = 14,11 (β-arrestin2−/−).

* $P < 0.05$ compared with control (same genotype).

† $P < 0.05$ compared with WT (same cage condition).

FC, fragmented maternal care; WT, wild-type.

3.4. Fragmented care affects the abundance of transcripts encoding proteins in the opioid pathway

Opioid peptides, receptors, and other components of the opioid signalling pathway influence nociception and responses to morphine.[42] We used quantitative PCR to examine the expression of transcripts for opioid peptide precursors, opioid receptors, and β-arrestin2, in juvenile (PD 11-18) and adult (PD 60) mice exposed to normal care and FC. The transcripts examined encode proopiomelanocortin (*Pomc*), proenkephalin (*Penk*), *Pdyn*, μ (*Oprm1*), δ (*Oprd1*), and κ (*Oprk1*) opioid receptors, and β-arrestin2 (*Arrb2*). We extracted RNA from spinal cord, the prefrontal cortex, thalamus, hypothalamus, hippocampus, and brainstem. Data and accompanying statistics from spinal cord are shown in **Figure 3** and in **Tables 2 and 3** (juvenile and adult mice, respectively). Statistics for the brain regions are also reported in **Tables 2 and 3**. Student *t*-test data, for each comparison, are reported in Supplemental Tables 1 and 2, available at http://links.lww.com/PAIN/B818 (juvenile and adult mice, respectively).

Prior FC produced tissue- and age-dependent effects on the expression of several components of the opioid pathway in mice (**Tables 2,3**). The most extensive changes in expression of opioid-related transcripts occurred in the spinal cord (**Figs. 3A and B**) and brainstem (**Table 2**). Data from juvenile (PD 11-18) and adult (PD 60) mice reveal that FC consistently increased the expression of *Oprd1 and Pdyn* transcripts in the spinal cord, whereas the expression of Pomc transcripts within this region were consistently reduced (**Figs. 3A and B**). The expression of Arrb2 transcripts increased in the spinal cords of juvenile mice but decreased in adult mice after FC. Furthermore, while FC did not affect the expression of Oprk1 transcript in the spinal cord of juvenile mice, expression was decreased in adult mice. There was no significant change in the expression of any other tested transcripts at either age in the spinal cords of FC mice.

Together, these data demonstrate that exposure to FC alters the expression of mRNAs transcribed by genes encoding opioid peptides, receptors, and signal transduction mediators in the spinal cord and brains of mice.

3.5. δ receptors and β-arrestin2 are required for the impact of fragmented maternal care on nociception and morphine tolerance

We examined whether δ receptors are required for the effects of FC on nociception, morphine antinociception, and tolerance using δ−/− mice. Consistent with this hypothesis, there was neither a difference in tail withdrawal latencies from warm water nor the threshold for mechanical withdrawal in FC compared with control δ−/− mice (**Figs. 4A and B**). Tail withdrawal latencies (mean ± SEM) were 3.4 ± 0.2 seconds and 3.5 ± 0.2 seconds in control and FC δ−/− mice, respectively, while mechanical thresholds (mean ± SEM) were 4.3 ± 0.1 g in control and 4.0 ± 0.1 g in FC δ−/− mice. In addition, there was no apparent shift in the morphine dose–response relationships in δ−/− mice exposed to FC compared with control δ−/− mice on day 1 (**Fig. 4C**; **Table 1**) or on day 10 (**Table 1**). There was also no significant impact of FC on $logED_{50}$ values on day 1 or on day 10 (**Table 1**). Furthermore, in contrast to WT mice, in which exposure

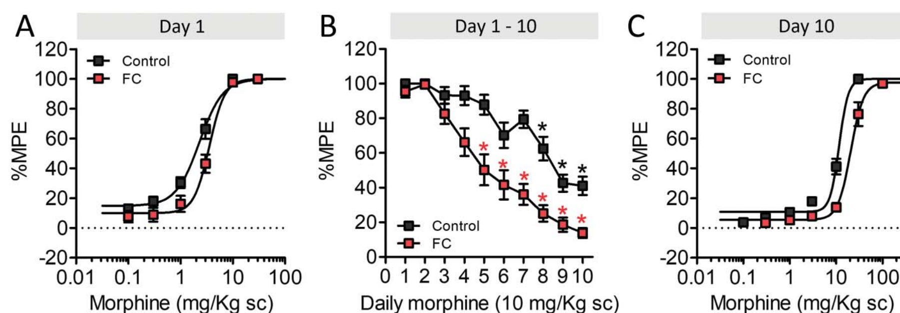

Figure 2. Fragmented care diminishes morphine antinociception and accelerates the development of tolerance in WT mice. Morphine antinociception in WT C57BL/6 control and fragmented care (FC) mice was assessed on postnatal day (PD) 60 using the warm-water (48°C) tail withdrawal assay. (A) Morphine cumulative dose–response relationship in control and FC mice on day 1. (B) Morphine (10 mg/kg) antinociception continued to be assessed using the tail withdrawal assay for the following 8 consecutive days. Separate Friedman tests comparing the maximum possible effect (%MPE) of morphine antinociception to day 1 within each group revealed a significant reduction in morphine antinociception after 8 days of injections in control mice ($\chi^2(9) = 94.1$, $P < 0.0001$). By contrast, morphine antinociception was significantly reduced in FC after just 5 days of injections ($\chi^2(9) = 92.9$, $P < 0.0001$). (C) Morphine cumulative dose–response relationships in control and FC mice on day 10. Estimates of morphine ED_{50} in control and FC mice on day 1 and on day 10 are summarised in Table 1. Data shown as mean ± SEM in all cases. Control: n = 16 (comprised 8 males and 8 females), and FC: n = 15 (comprised 7 males and 8 females). *$P <$ 0.05 compared with day 1. WT, wild-type.

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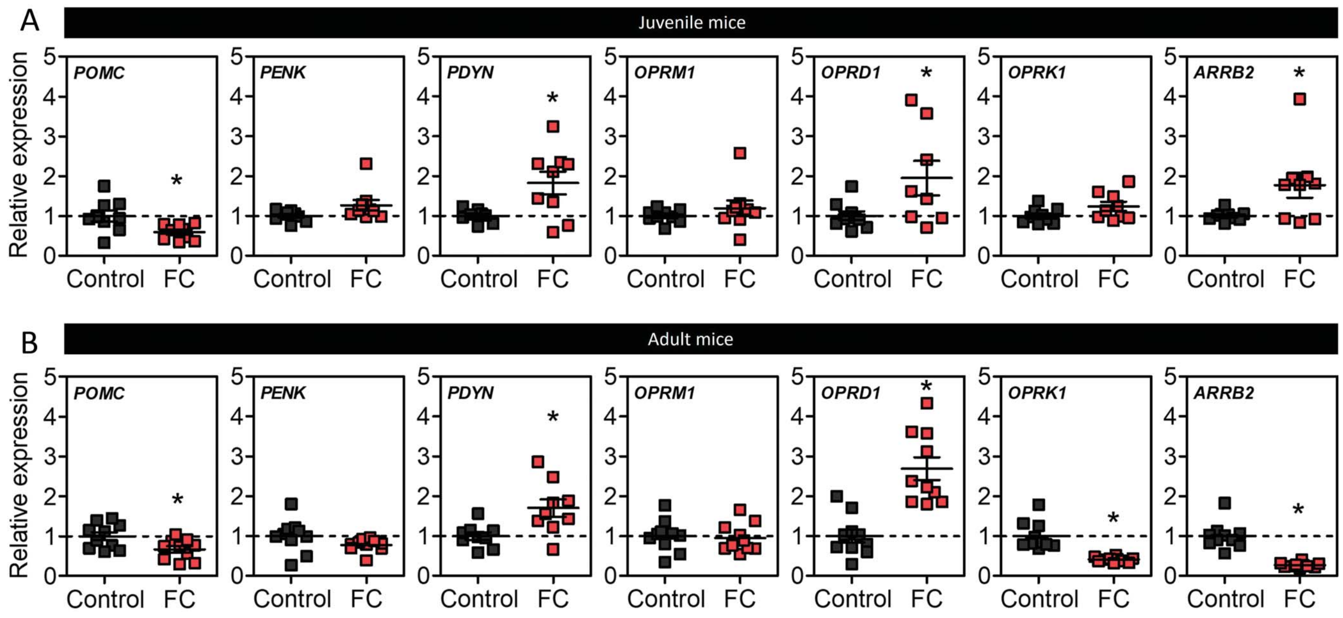

Figure 3. Fragmented care alters the expression of opioid pathway genes in the spinal cord of juvenile and adult C57BL/6 mice. Quantitative PCR was used to measure the expression of transcripts involved in opioid signalling (see Methods) in the spinal cords of (A) juvenile (postnatal day 11-18) and (B) adult (postnatal day 60) male and female WT C57BL/6 mice previously housed in control or fragmented care cages (see Methods). (A) Exposure to FC reduced the expression of Pomc ($P = 0.015$) mRNA and enhanced the expression of Pdyn ($P = 0.017$), Oprd1 ($P = 0.042$), and Arrb2 ($P = 0.027$) mRNAs in the spinal cord of juvenile mice relative to controls. (B) Adult mice previously exposed to FC similarly had enhanced expression of Pdyn ($P = 0.01$) and Oprd1 ($P = 0.0005$) mRNAs, and reduced expression of Pomc ($P = 0.019$) mRNA, in the spinal cord relative to control mice, whereas Arrb2 mRNA expression was significantly reduced ($P < 0.0001$). Data are normalised to GAPDH expression using the $2^{-\Delta\Delta CT}$ method and expressed relative to control mice for each age separately. Each datapoint represents mRNA expression from an individual mouse with the group mean and SD shown. Juvenile mice: n = 9 (control and FC), and adult mice: n = 10 (control and FC). *$P < 0.05$ compared with control. FC, fragmented maternal care; WT, wild-type.

to FC hastened the onset of morphine tolerance (**Fig. 2B**), no impact was observed for δ−/− mice (**Fig. 4D**). These data reveal that the shift in ED_{50} caused by 10 days exposure of δ−/− mice to morphine (**Table 1**) was not significantly affected by FC, unlike our observations in WT mice. Therefore, tolerance is not reinstated by exposure to FC in δ−/− mice.

Our previous study implicates β-arrestin2 in mediating the behavioural effects of enhanced δ receptor expression caused by exposure to inflammatory sensitivity.[35] We tested β-arrestin2−/− mice to establish whether β-arrestin2 is also required for the effects of FC on nociception and morphine antinociception and tolerance. Consistent with this hypothesis, FC did not affect the tail withdrawal latencies from warm water (mean latencies with SEM of 6.2 ± 0.6 seconds and 6.6 ± 1.0 seconds in control and FC β-arrestin2−/− mice, respectively), nor did FC affect the threshold for mechanical withdrawal (4.4 ± 3.0 g) compared with control (4.4 ± 0.2 g) β-arrestin2−/− mice (**Figs. 4E and F**). There was also no apparent shift in the dose–response relationships in β-arrestin2−/− mice exposed to FC compared with control β-arrestin2−/− mice on day 1 (**Fig. 4G**; **Table 1**) or on day 10 (**Table 1**). Furthermore, the MPE of morphine was sustained across the 10 days in both control and FC β-arrestin2−/− mice (**Fig. 4H**).

Importantly, neither the absence of δ receptors nor β-arrestin2 affected the impact of limited bedding on either dam sorties or fluctuations in the body weights of offspring (Supplementary Figure 2A-H, available at http://links.lww.com/PAIN/B818; **Fig. 5**). Taken together, these data demonstrate that δ receptors and β-arrestin2 are required for the impact of fragmented care on nociception, morphine antinociception, and tolerance.

Table 2

Fragmented maternal care alters the abundance of transcripts encoding opioid peptides, opioid receptors, and β-arrestin2 in the spinal cord and brains of juvenile mice.

	Pomc	Penk	Pdyn	Oprm1	Oprd1	Oprk1	Arrb2
Spinal cord	0.6 ± 0.2*	1.3 ± 0.4	1.8 ± 0.9*	1.2 ± 0.6	1.9 ± 1.2*	1.2 ± 0.3	1.8 ± 0.9*
Brainstem	1.6 ± 1.9	2.1 ± 1.1*	1.1 ± 0.5	1.5 ± 0.4*	3.1 ± 2.2*	1.5 ± 0.4*	1.2 ± 0.4
PFC	0.8 ± 0.2	0.6 ± 0.4	1.0 ± 0.3	0.8 ± 0.2	1.1 ± 0.6	0.7 ± 0.4	1.2 ± 0.2
Thalamic axis	1.0 ± 1.2	1.1 ± 0.7	0.9 ± 0.5	0.9 ± 0.3	1.4 ± 0.8	0.3 ± 0.1*	0.8 ± 0.4
Hippocampus	0.9 ± 0.6	1.0 ± 0.4	0.8 ± 0.3	1.2 ± 0.3	1.5 ± 1.1	1.1 ± 0.4	0.8 ± 0.3

Quantitative PCR was performed on RNA extracted from the spinal cord, brainstem, prefrontal cortex, thalamic axis, and hippocampus of juvenile (postnatal day 11-18) mice previously exposed to control or fragmented care cages (see Methods). The Student *t*-test data for each gene expression comparison are summarised in Supplementary Table 1, available at http://links.lww.com/PAIN/B818. Data are the mean expression (with SD) in FC mice expressed relative to the corresponding gene in each region in control mice and are normalised to GAPDH expression using the $2^{-\Delta\Delta CT}$ method.

* $P < 0.05$ compared with control expression.

PFC, prefrontal cortex.

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Table 3

Fragmented maternal care alters the abundance of transcripts encoding opioid peptides, opioid receptors, and β-arrestin2 in the spinal cord and brains of adult mice.

	Pomc	Penk	Pdyn	Oprm1	Oprd1	Oprk1	Arrb2
Spinal cord	0.7 ± 0.3*	0.8 ± 0.2	1.8 ± 1.2*	1.0 ± 0.4	3.0 ± 2.1*	0.4 ± 0.1*	0.3 ± 0.1*
Brainstem	0.5 ± 0.2*	0.9 ± 0.3	0.7 ± 0.3	1.2 ± 0.8	1.3 ± 0.9	0.9 ± 0.4	0.9 ± 0.2
PFC	0.9 ± 0.2	0.7 ± 0.7	2.5 ± 1.3*	0.9 ± 0.6	1.0 ± 0.4	0.6 ± 0.1*	1.5 ± 1.1
Thalamus	0.7 ± 0.3	1.1 ± 0.7	0.5 ± 0.3	0.4 ± 0.1*	0.7 ± 0.6	0.8 ± 0.3	1.4 ± 0.6
Hypothalamus	0.8 ± 0.5	0.7 ± 0.2	0.8 ± 0.4	1.1 ± 0.2	0.7 ± 0.3	0.9 ± 0.4	1.0 ± 0.5
Hippocampus	0.8 ± 0.4	0.9 ± 0.3	0.8 ± 0.4	0.8 ± 0.3	1.5 ± 0.5*	0.9 ± 0.4	0.5 ± 0.2*

Quantitative PCR was performed on RNA extracted from the spinal cord, brainstem, prefrontal cortex, thalamus, hypothalamus, and hippocampus of adult (postnatal day 60) mice previously exposed to control or fragmented care cages (see Methods). The Student *t*-test data for each gene expression comparison are summarised in Supplementary Table 2, available at http://links.lww.com/PAIN/B818. Data are the mean expression (with SD) in FC mice expressed relative to the corresponding gene in each region in control mice and are normalised to GAPDH expression using the $2^{-\Delta\Delta CT}$ method.

* $P < 0.05$ compared with control expression.

PFC, prefrontal cortex.

3.6. Fragmented care causes persistent mechanical hypersensitivity that is dependent on δ receptors and β-arrestin2

Administration of CFA into a hind paw causes the development of transient mechanical hypersensitivity in WT mice, which resolves through enhanced opioid receptor signalling.[7,35,40] We assessed the development of mechanical hypersensitivity evoked by CFA in adult WT mice exposed to FC and controls (**Fig. 6A**). Absolute withdrawal thresholds (Supplementary Table 3, available at http://links.lww.com/PAIN/B818) were expressed relative to baseline in each mouse to establish the impact of FC on mechanical hypersensitivity while accounting for differences in basal mechanical nociception (**Fig. 1D**). This is important because β-arrestin2−/− mice have reduced acute nociception due to enhanced μ receptor activity.[3,18,41]

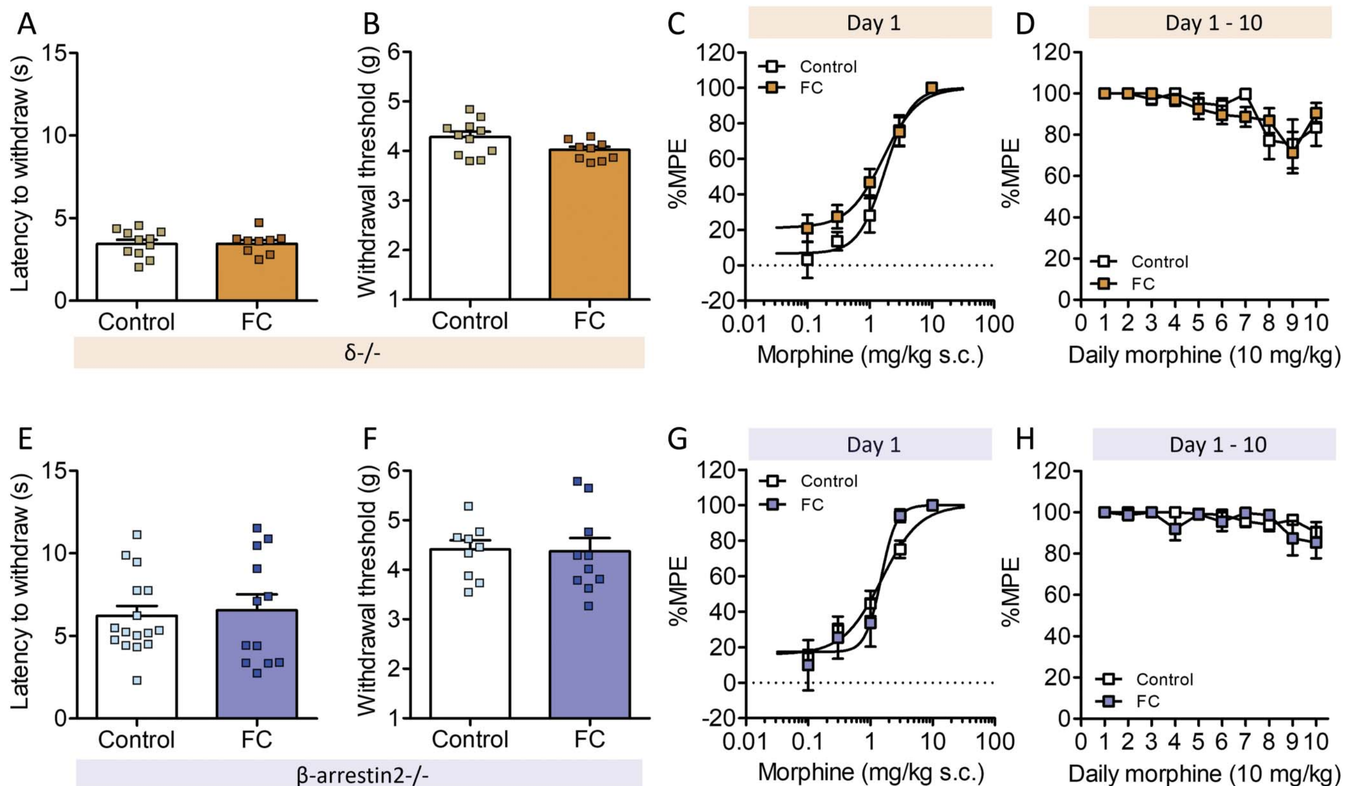

Figure 4. Fragmented care has no effect on acute nociception or morphine antinociception in δ−/− and β-arrestin2−/− mice. Acute thermal nociception and morphine antinociception were assessed using the warm water (48˚C) tail withdrawal assay in adult (postnatal day 60) control or fragmented care (FC) δ−/− and β-arrestin2−/− mice (see Supplementary Fig. 2, available at http://links.lww.com/PAIN/B818). Mechanical nociception was assessed using automated von Frey. (A and E) FC had no effect on acute thermal nociception in (A) δ−/− ($P = 0.98$) or (E) β-arrestin2−/− ($P = 0.76$) mice. (B and F) FC similarly had no effect on acute mechanical nociception in either (B) δ−/− ($P = 0.1$) or (F) β-arrestin2−/− ($P = 0.92$) mice. (C and G) Morphine cumulative dose–response relationships in control and FC δ−/− (C) and β-arrestin2−/− (G) mice on day 1. (D and H) Morphine (10 mg/kg) antinociception continued to be assessed using the tail withdrawal assay for the following 8 consecutive days in control and FC δ−/− (D) and β-arrestin2−/− (H) mice. Separate Friedman tests with Bonferroni corrections comparing the maximum possible effect (%MPE) of morphine antinociception to day 1 within each group detected no significant reduction in morphine antinociception in either control or FC (D) δ−/− or (H) β-arrestin2−/− mice. Estimates of morphine ED_{50} in control and FC δ−/− and β-arrestin2−/− mice on day 1 and on day 10 are summarised in Table 1. Data shown as mean ± SEM in all cases. δ−/− mice: n = 8 (control) and 11 (FC), and β-arrestin2−/− mice: n = 14 (control) and 11 (FC).

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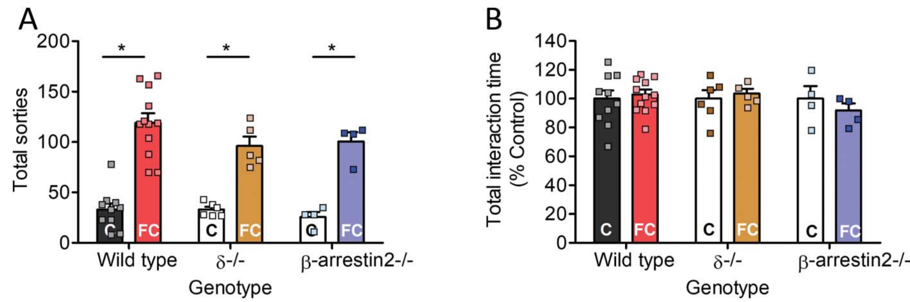

Figure 5. Fragmented care paradigm is not obviously impaired in the absence of δ receptors or β-arrestin2. (A) Total number of nest departures (termed sorties) made by C57BL/6 WT, δ−/−, and β-arrestin2−/− control or fragmented care dams (Fig. 1A and Supplementary Figs. 2A and E, available at http://links.lww.com/PAIN/B818) during postnatal days (PD) 3 to 8. A 2-way ANOVA comparing the total number of sorties, summed across all days with genotype and cage conditions as factors, detected a significant effect of cage condition ($F_{1,35}$ = 89.3, P < 0.0001), although there was no significant effect of genotype ($F_{2,35}$ = 1.4, P = 0.26) or an interaction ($F_{2,35}$ = 0.9, P = 0.4). (B) Total interaction time between control or FC WT, δ−/−, and β-arrestin2−/− dams and their pups (Fig. 1B and Supplementary Figs. 2B and F, available at http://links.lww.com/PAIN/B818) during PD 3 to 8. A 2-way ANOVA comparing interaction times with genotype and cage conditions as factors detected no significant effect of genotype ($F_{2,35}$ = 0.54, P = 0.59), cage condition ($F_{1,35}$ = 0.02, P = 0.89), or an interaction ($F_{2,35}$ = 0.54, P = 0.59). Together, these data demonstrate that fragmented care caused by limited bedding in WT mice is not impaired in δ−/− or β-arrestin2−/− mice. WT mice: n = 10 and 12 (separate control and FC cages, respectively), δ−/− mice: n = 6 and 5 (separate control and FC cages, respectively), and β-arrestin2−/− mice: n = 4 and 4 (separate control and FC cages, respectively). ANOVA, analysis of variance; C, control; FC, fragmented maternal care; WT, wild-type.

Injection with CFA into one hind paw (see Methods) caused mechanical hypersensitivity to develop in WT control and FC mice (**Fig. 6A**). Mechanical thresholds in the ipsilateral paws were reduced from baseline by injection with CFA for up to 7 days in both control and FC mice. Although recovery back to baseline sensitivity was complete by day 7 and sustained until day 30 in WT control mice, mechanical hypersensitivity recurred from day 11 in mice exposed to FC (**Fig. 6A**). The mechanical thresholds of FC mice were significantly reduced compared with those of control mice on day 30 (**Fig. 6A**; Supplementary Table 3, available at http://links.lww.com/PAIN/B818). Importantly, no change in mechanical sensitivity occurred in the paw contralateral to the injection site throughout the 30-day assessment (Supplementary Figure 3, available at http://links.lww.com/PAIN/B818). These data demonstrate that FC causes mechanical hypersensitivity to recur in the inflamed paw of WT mice and become persistent.

We used the same approach to assess the development of CFA-evoked mechanical hypersensitivity in control or FC δ−/− and β-arrestin2−/− mice. Mechanical thresholds were reduced by injection with CFA for up to 15 days in control δ−/− (**Fig. 6B**) and for up to 9 days in control β-arrestin2−/− mice (**Fig. 6C**). In contrast to the effect of FC in WT mice, FC in δ−/− or β-arrestin2−/− mice had no effect on the recovery from mechanical hypersensitivity. Mechanical sensitivities on day 30 in either control or FC δ−/− and β-arrestin2−/− mice were similar to their sensitivities at baseline (Supplementary Table 3, available at http://links.lww.com/PAIN/B818). Together, these data demonstrate that FC does not alter the trajectory for normal recovery from mechanical hypersensitivity, nor does it establish persistent mechanical hypersensitivity, in the absence of δ receptors or β-arrestin2.

4. Discussion

Fragmented maternal care reduced acute thermal and mechanical sensitivities and the apparent potency of morphine, while accelerating morphine tolerance. These effects were

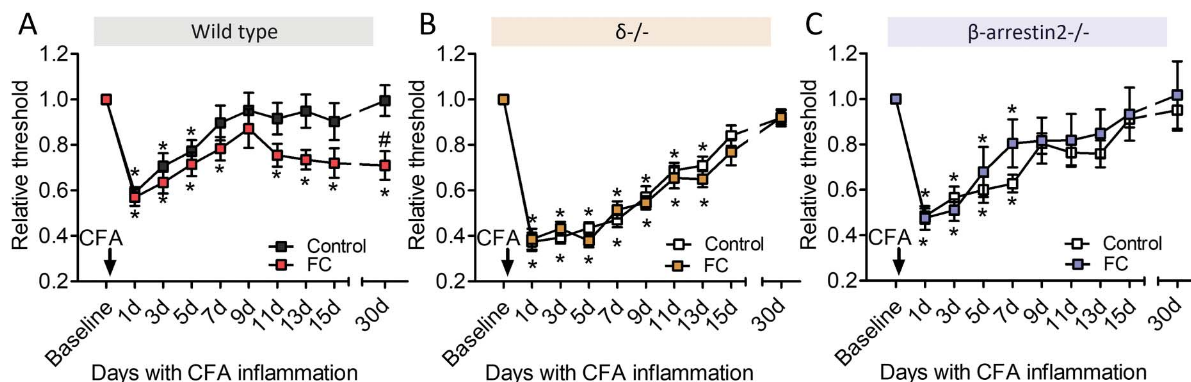

Figure 6. Fragmented care causes persistent mechanical hypersensitivity that is dependent on δ receptors and β-arrestin2. Mechanical sensitivities of control and fragmented care (FC) WT (A), δ−/− (B), and β-arrestin2−/− (C) mice, expressed relative to baseline, were assessed for 30 days after a unilateral injection with 10-μL undiluted (1 mg/mL) complete Freund's adjuvant (CFA) in 1 hind paw. Statistical comparisons were performed using a 2-way ANOVA with repeated measures for each genotype separately using cage condition and time as factors. (A) Recovery from CFA-evoked hypersensitivity was complete and sustained by day 7 in WT control mice. By contrast, whereas recovery occurred by day 7 in WT FC mice, mechanical hypersensitivity recurred in these mice on day 11 and beyond (time: $F_{9,216}$ = 9.8, P < 0.0001; cage condition: $F_{1,24}$ = 4.3, P = 0.05; interaction: $F_{9,216}$ = 2.0, P = 0.04). FC did not affect recovery in (B) δ−/− or (C) β-arrestin2−/− mice. Recovery occurred by day 15 in δ−/− mice (time: $F_{9,162}$ = 70.5, P < 0.0001; cage condition: $F_{1,18}$ = 0.3, P = 0.57; interaction: $F_{9,162}$ = 0.8, P = 0.65) and by day 9 in β-arrestin2−/− mice (time: $F_{9,153}$ = 25.3, P < 0.0001; cage condition: $F_{1,17}$ = 0.3, P = 0.61; interaction: $F_{9,153}$ = 0.8, P = 0.59) and was sustained on day 30. Data are expressed as mean ± SEM. Absolute mechanical sensitivities are summarised in Supplementary Table 3 (available at http://links.lww.com/PAIN/B818). *P < 0.05 compared with baseline, and #P < 0.05 compared with control. n (control, then FC) = 13 and 13 (WT), n = 9 and 11 (δ−/−), and n = 9 and 10 (β-arrestin2−/−). WT, wild-type.

accompanied by changes in expression of opioid pathway genes in juvenile (PD 11-18) and adult (PD ≥ 60) mice exposed to FC. Some changes were age-dependent; however, elevated δ receptor expression in the spinal cord was a consistent phenomenon. Furthermore, FC increased persistent inflammatory mechanical hypersensitivity in male and female mice. Fragmented maternal care–associated alterations in basal nociception, inflammatory sensitivity, and responses to morphine were not seen in δ−/− and β-arrestin2−/− mice, suggesting δ receptors and β-arrestin2 are required for these effects of exposure to neonatal adversity.

It has been established that β-arrestin2−/− mice have reduced basal nociception compared with WT mice, through enhanced μ receptor constitutive activity,[3,18] and this could diminish a further reduction by FC. However, this is not the case for δ−/− mice, which do not have enhanced basal nociception[35] and show no effect of FC.

Consistent with previous studies,[12,31] FC caused reduced body weight during development, which recovered by PD 60. Importantly, neither FC in response to limited bedding nor the impact of FC on body weights was affected by the absence of either δ receptors or β-arrestin2.

Fragmented maternal care–induced changes in the expression of opioid pathway genes might contribute to altered nociception and morphine responses. Fragmented maternal care altered the expression of 4 transcripts in the spinal cords of juveniles and 5 were affected in adults. Three changes in expression were conserved between both age groups. The brainstem was the next most affected region, with changes in juvenile mice in 4 of the 7 transcripts tested. However, only one FC-associated change was observed in the brainstems of adult mice, and this was not conserved between the 2 age groups. Therefore, we focus here primarily on gene expression in the spinal cord. The impact of FC on gene expression in the prefrontal cortex, hippocampus, thalamic axis, and brainstem is considered in the Supplementary Discussion, available at http://links.lww.com/PAIN/B818.

Fragmented maternal care caused changes in the expression of 2 of the 3 genes that encode opioid peptide precursors in juvenile and adult mice. Fragmented maternal care decreased *Pomc* and increased *Pdyn* expression in the spinal cord. Proopiomelanocortin is particularly pertinent as it is the precursor of several biologically active peptides including adrenocorticotrophin and the opioid, β-endorphin. Although there is evidence for POMC-expressing cell bodies in the rodent spinal cord, their role in nociception has not been established.[13] By contrast, the importance of PDYN in the spinal cord as a potential biomarker of pain is well established. Chronic pain is associated with increased *Pdyn* expression and elevated dynorphin.[29] Furthermore, intrathecal dynorphin peptide and antidynorphin antibody initiates allodynia and decreases nociception, respectively. Also, elevated spinal dynorphin increases morphine antinociceptive tolerance, which is diminished by spinal dynorphin antiserum.[37] Therefore, enhanced *Pdyn* expression in the spinal cords of mice exposed to FC may contribute to enhanced morphine tolerance and persistent inflammatory hypersensitivity. These effects appear to be independent of κ receptors and may be mediated by NMDA receptors.[29,37]

Prodynorphin-positive GABAergic interneurons in the mouse dorsal horn suppress mechanosensation and itch during adulthood and they transition from phasic to tonic firing during the neonatal period.[2] The effect of FC on *Pdyn* expression in juvenile and adult mice may indicate an impact in this important population of neurons.

In addition to altering the expression of opioid peptide precursors, FC caused changes in the expression of genes encoding opioid receptors. Although there was no change in *Oprm1* expression, the expression of *Oprk1* was reduced in the spinal cord, which may be an adaptive response to the FC-induced increase in *Pdyn* expression. However, enhanced *Pdyn* mRNA caused by peripheral inflammation does not result in altered spinal κ receptor expression.[16] On this basis, it is perhaps more likely that FC causes reduced *Oprk1* through a mechanism independent of elevated dynorphin. Activation of spinal κ receptors causes antinociception.[29] Therefore, if the observed reduction in *Oprk1* expression translates to fewer spinal κ receptors, this may contribute to FC-evoked persistent inflammatory hypersensitivity.

Fragmented maternal care elevated *Oprd1* transcript in the spinal cord. Most δ receptors are expressed by lamina II excitatory interneurons involved in the transmission of noxious mechanical stimuli.[30] In some cases, δ and μ receptors are expressed in the same neurons, in which they may interact, perhaps forming heteromers.[45] Increased *Oprd1* expression after exposure to FC may drive enhanced association of δ and μ receptors and this may contribute to *Oprd1*-dependent behavioural effects of FC. The use of δ−/− mice revealed a requirement for δ receptors in FC-evoked hyposensitivity. Elevated expression induced by FC may contribute to reduced basal nociception through greater opioid tone mediated by δ receptors and/or μ-δ heteromers.

It is important to acknowledge that, although quantitative PCR data provide important insights into altered transcript levels, additional studies will be required to establish whether FC alters protein expression and influences the opioid signalling pathway.

Consistent with a role for δ receptors in reducing nociception, recovery from CFA-induced hypersensitivity is slowed by an absence of δ receptors.[35,40] Furthermore, CFA-evoked inflammation upregulates δ receptor transcript in the spinal cord, suggesting that increased spinal δ expression contributes to reduced hypersensitivity.[35] However, our previous study demonstrated that δ receptors are required for morphine-induced hypersensitivity after CFA-evoked inflammation in WT mice. Similarly, in the current study, expression of δ receptors was required for the transition from recovery to persistent inflammatory hypersensitivity seen in mice exposed to FC, but not in control mice. These findings suggest that, although an upregulation of δ receptor expression may provide an initial reduction in nociception and relief from inflammatory hypersensitivity, this can transition to persistent hypersensitivity. We hypothesise that after a short-lived protective effect, upregulation of δ receptor expression in those exposed to early-life adversity increases vulnerability to the development of persistent pain.

The expression of μ receptors is essential for recovery from CFA-evoked hypersensitivity.[7,35,40] FC- and CFA-evoked inflammation, which both stimulate enhanced δ receptor transcript in the spinal cord, may increase association of δ receptors with μ receptors, leading to increased constitutive recruitment of β-arrestin2. Such an outcome, while providing temporary hyposensitivity, might prime neurons for heightened opioid tolerance and hypersensitivity.[35] The observation that β-arrestin2−/− mice exhibited no effect of FC on either morphine tolerance or persistent mechanical hypersensitivity after CFA is consistent with the hypothesis that β-arrestin2 mediates these effects. As well as being dependent on δ receptor expression, morphine-induced inflammatory hypersensitivity after CFA also requires β-arrestin2.[35] Importantly, our previous study demonstrated that neither a lack of δ receptors nor β-arrestin2 affects

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either the inflammatory response to CFA or acute hypersensitivity. Taken together, these findings establish that FC increases morphine tolerance and persistent inflammatory hypersensitivity through a δ receptor– and β-arrestin2–dependent process.

Fragmented maternal care decreased *Arrb2* expression in spinal cords of adult male and female mice. By contrast, FC increased *Arrb2* expression in juvenile mice. Reduced β-arrestin2 transcript in adults might contribute to reduced acute nociception. Depletion of β-arrestin2 in β-arrestin2−/− mice enhances agonist-independent μ receptor activity resulting in constitutive inhibitory coupling to voltage-activated Ca^{2+} channels and basal nociceptive hyposensitivity.[18,41] Our findings in β-arrestin2−/− mice, in which antinociception was not additionally enhanced by FC, supports a role of reduced β-arrestin2 expression within the spinal cord in reducing thermal and mechanical sensitivities. A reduction in β-arrestin2 expression may enhance inhibitory coupling of the μ receptor to confer the initial antinociception to mechanical and thermal stimuli.[41]

Based on most,[1,3,17,43] but not all,[14] prior studies, decreased β-arrestin2 expression would be expected to diminish morphine tolerance. However, we observed accelerated morphine tolerance in mice exposed to FC despite reduced β-arrestin2 expression in their spinal cords. Furthermore, there was no acceleration of morphine tolerance in β-arrestin2−/− mice exposed to FC. It is possible that the FC-associated reduction in β-arrestin2 transcript may not result in reduced protein. Alternatively, β-arrestin2 expression in the spinal cord may have a limited contribution to the development of antinociceptive tolerance. Indeed, evidence from conditional knockout mice in which the μ receptor was deleted in primary afferent neurons suggests that tolerance is initiated in nociceptors.[8]

While the discussion here has focused primarily on spinal mechanisms that may contribute to the impact of FC on nociception and hypersensitivity, there were several changes in gene expression in the brainstems (see Supplemental Discussion, available at http://links.lww.com/PAIN/B818) of juvenile mice. This adds to prior evidence for the involvement of the brainstem in the developmental influence of early-life adversity on pain.[9,39]

Taken together, these results suggest that FC causes bimodal effects on nociception, initially reducing acute sensitivity through enhanced opioid receptor activity. However, this is associated with enhanced morphine tolerance through increased δ receptor expression and recruitment of β-arrestin2. These processes increase vulnerability to the transition from acute to persistent hypersensitivity. These processes may also contribute to associations between adverse childhood experiences and chronic pain.[10] Our findings predict greater vulnerability to opioid tolerance in those exposed to early-life adversity.

Conflict of interest statement

The authors have no conflict of interest to declare.

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Data availability: The data that support the findings of this study are available from the corresponding author, upon reasonable request.

Appendix A. Supplemental digital content

Supplemental digital content associated with this article can be found online at http://links.lww.com/PAIN/B818.

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