Supplementary Materials

Sustained morphine exposure alters spinal NMDA receptor and astrocyte expression and exacerbates chronic pain behaviour in female rats

*S*ara V. Gonçalves[a,b,+], Stephen G. Woodhams[a,b,+], Li Li[a,b], Gareth J. Hathway[a,b,c], Victoria Chapman[a,b,c] *

[a] Pain Centre Versus Arthritis, University of Nottingham, Medical School, Queen's Medical Centre, Nottingham, [b] School of Life Sciences, Medical School, Queen's Medical Centre, Nottingham,
[c] NIHR Nottingham Biomedical Research Centre, University of Nottingham, Nottingham, United Kingdom

+ Joint first authors Sara Gonçalves & Stephen G Woodhams contributed equally to this work

* Corresponding author. Address: E177, School of Life Sciences, Medical School, Queen's Medical Centre, Nottingham, United Kingdom, NG7 2UH. Tel.: +44115 82 30136; Fax: +44115 82 30142. E-mail address: Victoria.chapman@nottingham.ac.uk.

Running Title: Morphine induced spinal plasticity and pain

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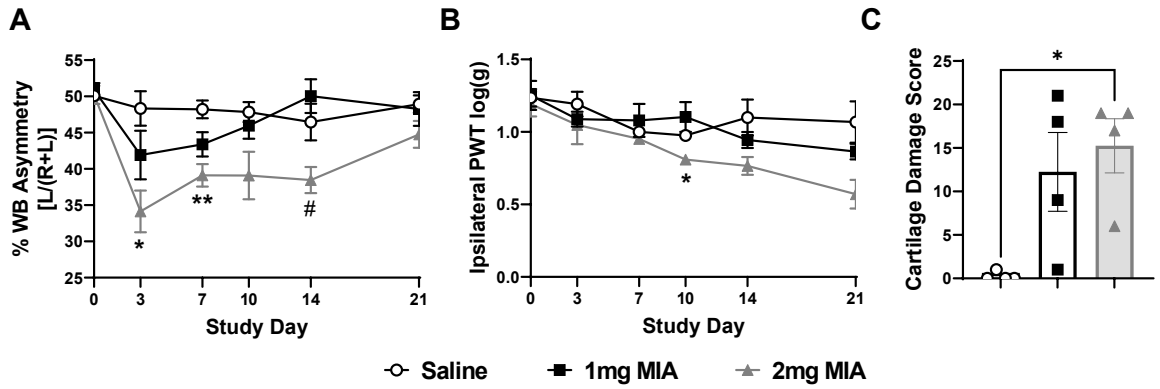

Figure S1 – OA-like pain and knee pathology in female rats induced with different doses of MIA. Female Sprague Dawley rats received intra-articular injection of either 1mg/50µl MIA (■), 2mg/50µl MIA (●) or 50µl saline (○) into the left knee at day 0 (n=4/group). Rats injected with 2mg MIA exhibited higher weight bearing asymmetry (**A**) compared to those injected with 1mg MIA. Injection of 2mg MIA induced a slight decrease in PWT (**B**), but only at later time points. Injection of 1mg MIA did not significantly reduce PWTs compared to injection of saline. Chondropathy was observed in both MIA-injected groups, but only 2mg MIA induced significant knee pathology when compared with saline controls.

Data are presented as mean±SEM. *p=0.5, **p<0.01, versus saline; #p<0.05 versus 1mg MIA. RM 2-way ANOVA with Tukey's post-hoc testing. ANOVA, analysis of variance; MIA, monosodium iodoacetate; OA, osteoarthritis; PWTs, paw withdrawal thresholds.

Protein loading per sample	***Target Protein***	**S*uppliers***	**S*ource***	**D*ilution for WB***
30µg	IBA1	Novus/NB100-1028	Goat	1:1000
5~10µg	GFAP	Dako/Z0334	rabbit	1:2000
30µg	NMDAR1	abcam/ab109182	rabbit	1:1000
40µg	NMDAR2b	abcam/ab65783	rabbit	1:1000
	Beta-actin	Sigma-Aldrich /a5441	mouse	1:2000

Table S1 – Protein isolation and Western blotting.

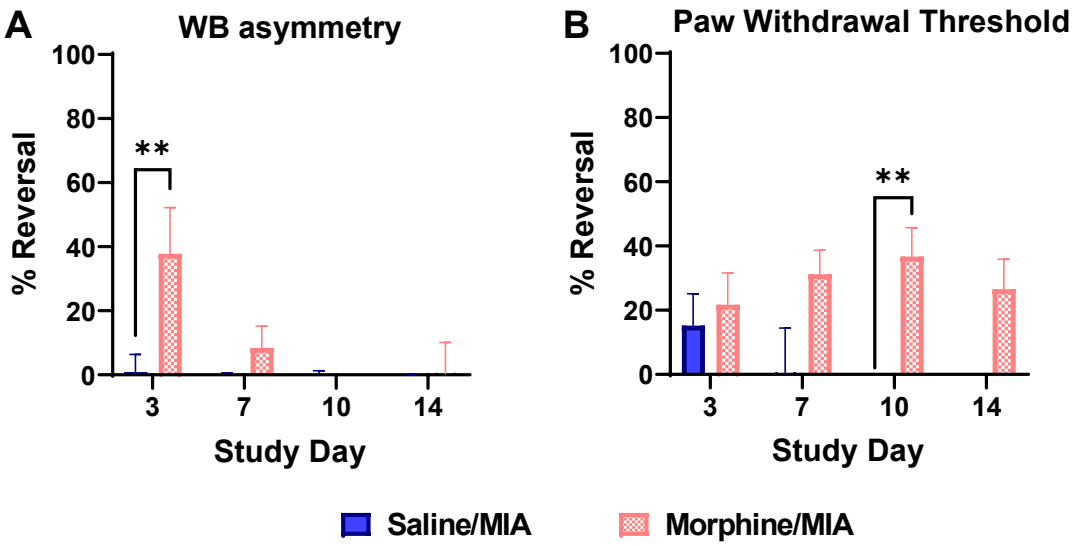

Figure S2 –Acute analgesic effects of morphine versus saline administration in the MIA model of OA-like pain in female rats. See figure 1A for timeline. To evaluate the acute analgesic effects of morphine treatment, pain behaviour was assessed 1hr after morning systemic injection. Morphine acutely reversed weight-bearing asymmetry (**A**) on day 3, but not at any later time points. No significant effect of drug treatment was observed, but there was a significant effect of time, and interaction between time and drug treatment (treatment: $F_{(1,9)}$=2.37, p=0.16; time: $F_{(1.7,15.3)}$=7.3, p=0.001; treatment x time: $F_{(3,27)}$=5.5, p=0.004, 2-way ANOVA). Šídák's multiple comparisons test revealed a significant difference between saline/MIA and morphine/MIA rats on day 3 only (p<0.01). Morphine also produced an acute, partial reversal of MIA-induced reductions in ipsilateral PWT (**B**), across the entire duration of the study (treatment: $F_{(1,10)}$=23.6, p=0.0007; time: $F_{(3,30)}$=0.27, p=0.85; treatment x time: $F_{(3,30)}$=1.5, p=0.25, 2-way ANOVA). Šídák's multiple comparisons test revealed a significant difference between saline/MIA and morphine/MIA rats on day 10 only (p<0.01). Data are presented as mean±SEM.

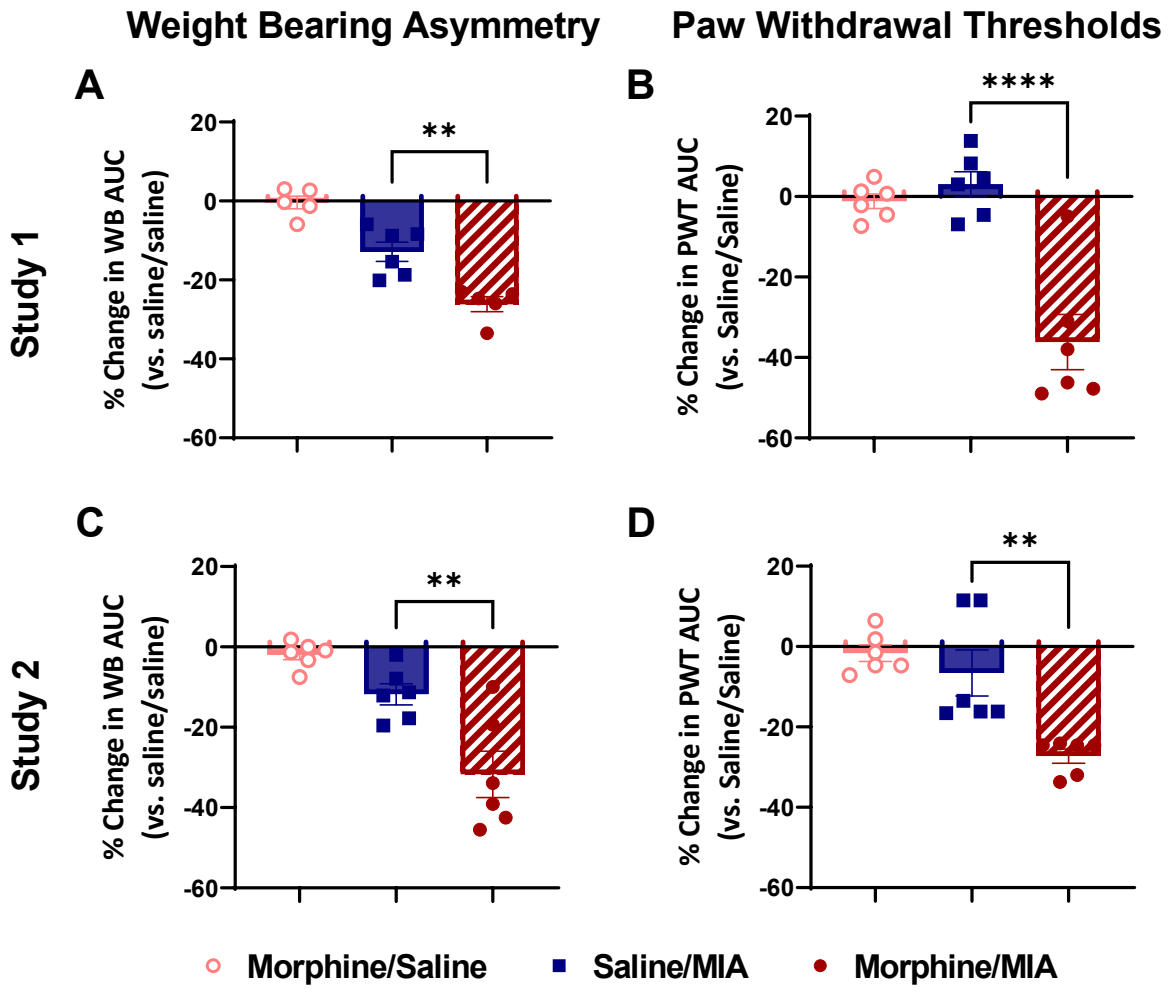

Figure S3 – Sustained exposure to morphine exacerbates weight bearing asymmetry and lowers PWTs in female rats to a similar degree 2 weeks after MIA injection in separate cohorts of animals. Area under the curve (AUC) data generated from time course data in figure 2, and expressed as % change compared to saline/saline. Unilateral intra-articular injection of 2mg MIA produced significant weight bearing asymmetry up to D14 in both study 1 (**A;** $F_{(2,13)}$=35.76, p<0.0001) and study 2 (**C;** $F_{(2,15)}$=16.66, p=0.0002), but no significant reduction in PWTs in either study (**B** & **D**). Sustained morphine exposure significantly exacerbated MIA-induced weightbearing asymmetry at D14 after MIA-injection in both study 1 (**A**) and study 2 (**C**), and substantially lowered PWTs (**B:** $F_{(2,15)}$=23.04, p<0.0001; **D:** $F_{(2,15)}$=13.78, p=0.0004). Sustained morphine exposure in the absence of the MIA model had no effect on either weight bearing or PWTs. These data confirm that sustained morphine exposure reproducibly exacerbates OA-like pain in the MIA model in female rats. Data are presented as mean ± SEM, n=6/group. **p<0.01, ****p<0.0001. One-way ANOVA with Tukey's *post-hoc* testing.

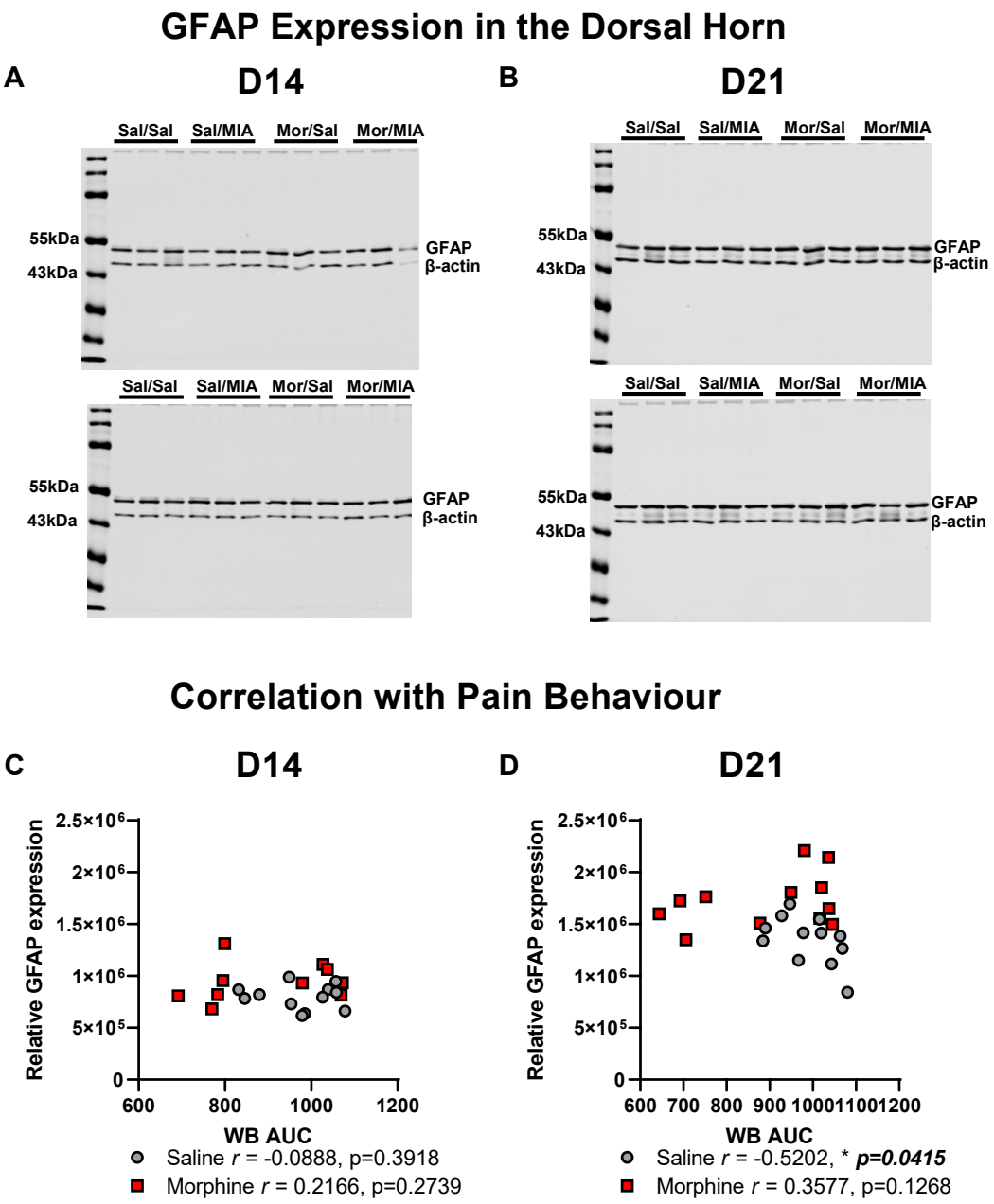

Figure S4 – GFAP expression in the spinal cord dorsal horn in a model of sustained opioid exposure and OA-like pain. Expanded full-length western blots from the panels shown in figure 4, depicting GFAP expression at D14 (**A**) and D21 (**B**) after intra-articular injection. No significant correlation was observed between weightbearing asymmetry AUC and spinal GFAP at D14 in study 1 (**C**). In contrast, spinal GFAP expression was significantly negatively correlated with weight bearing asymmetry at D21 in study 2 (**D**), but only in morphine-naïve rats.

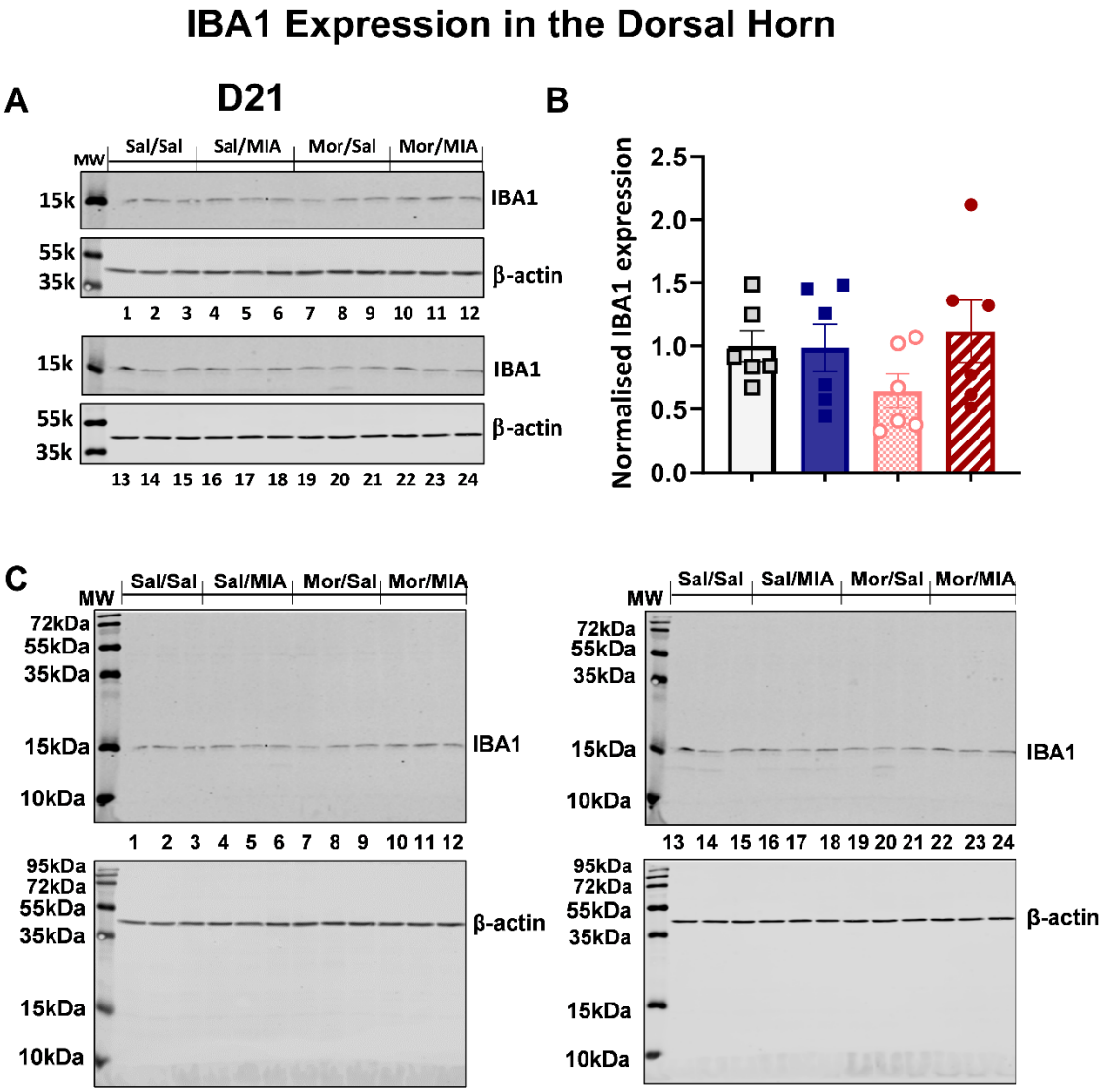

Figure S5 – IBA1 expression in the spinal cord dorsal horn is unaltered at D21 in a model of sustained opioid exposure and OA-like pain.

Spinal ipsilateral dorsal horn expression of IBA1 was similar in across all treatment groups at D21 after intra-articular injection (**A**, *n*=6/group). Quantification via densitometry showed no significant differences in IBA1 protein expression levels, suggesting microglia may not play a significant role in maintenance of chronic pain or the effects of sustained opioid exposure in this model (**B**). Expanded full-length western blots from panel **A** (**C**).

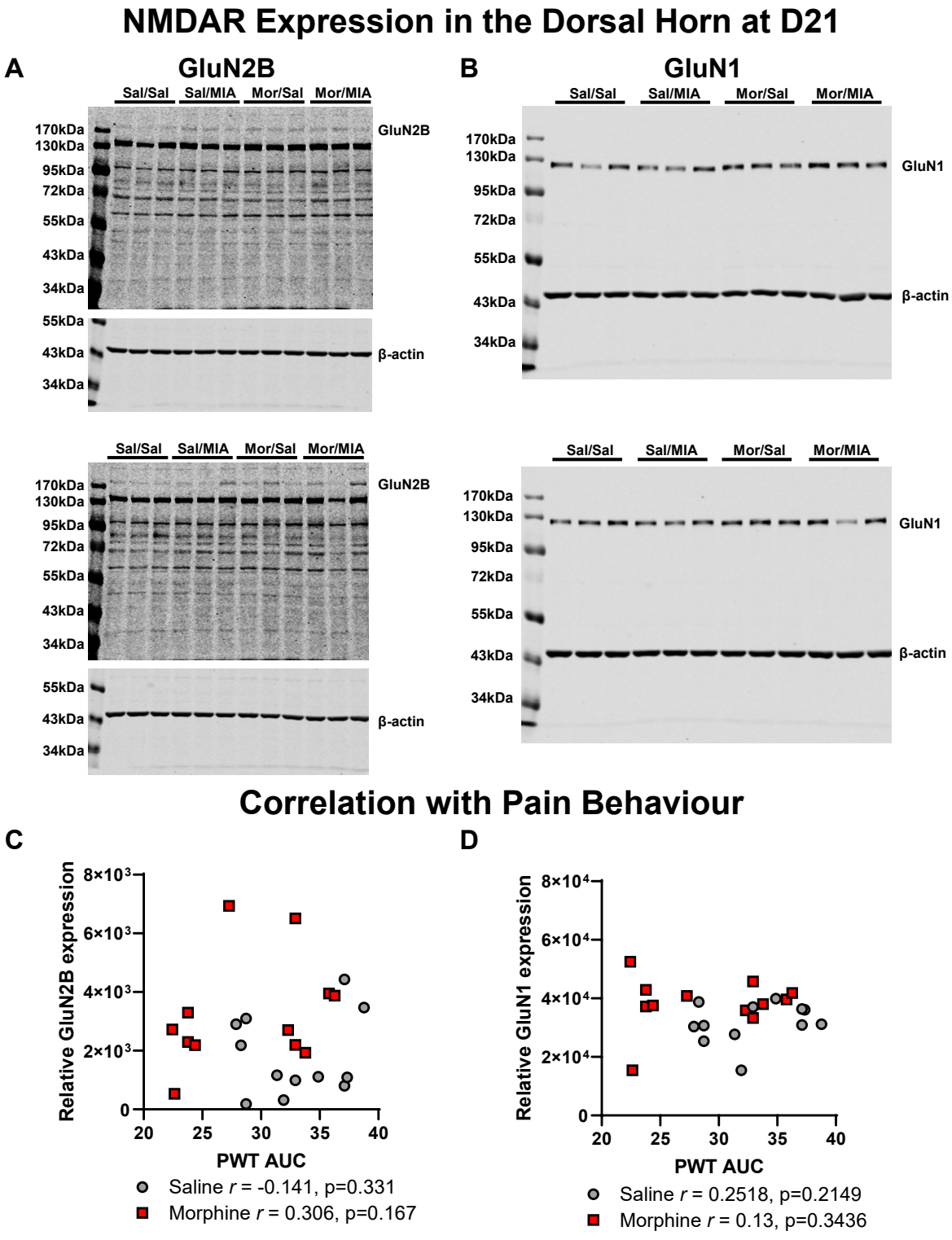

Figure S6 – NMDAR expression in the spinal cord dorsal horn in a model of sustained opioid exposure and OA-like pain. Expanded full-length western blots from the panels shown in figure 5, depicting spinal GluN2B (**A**) and GluN1 (**B**) expression after intra-articular injection. Spinal expression of GluN2B (**C**) or GluN1 (**D**) was not significantly correlated with PWT AUC at D21 in study 2. Correlations assessed via Pearson's correlation coefficient r.