Figures and figure supplements

Simultaneous brain, brainstem, and spinal cord pharmacological-fMRI reveals involvement of an endogenous opioid network in attentional analgesia

Valeria Oliva *et al*

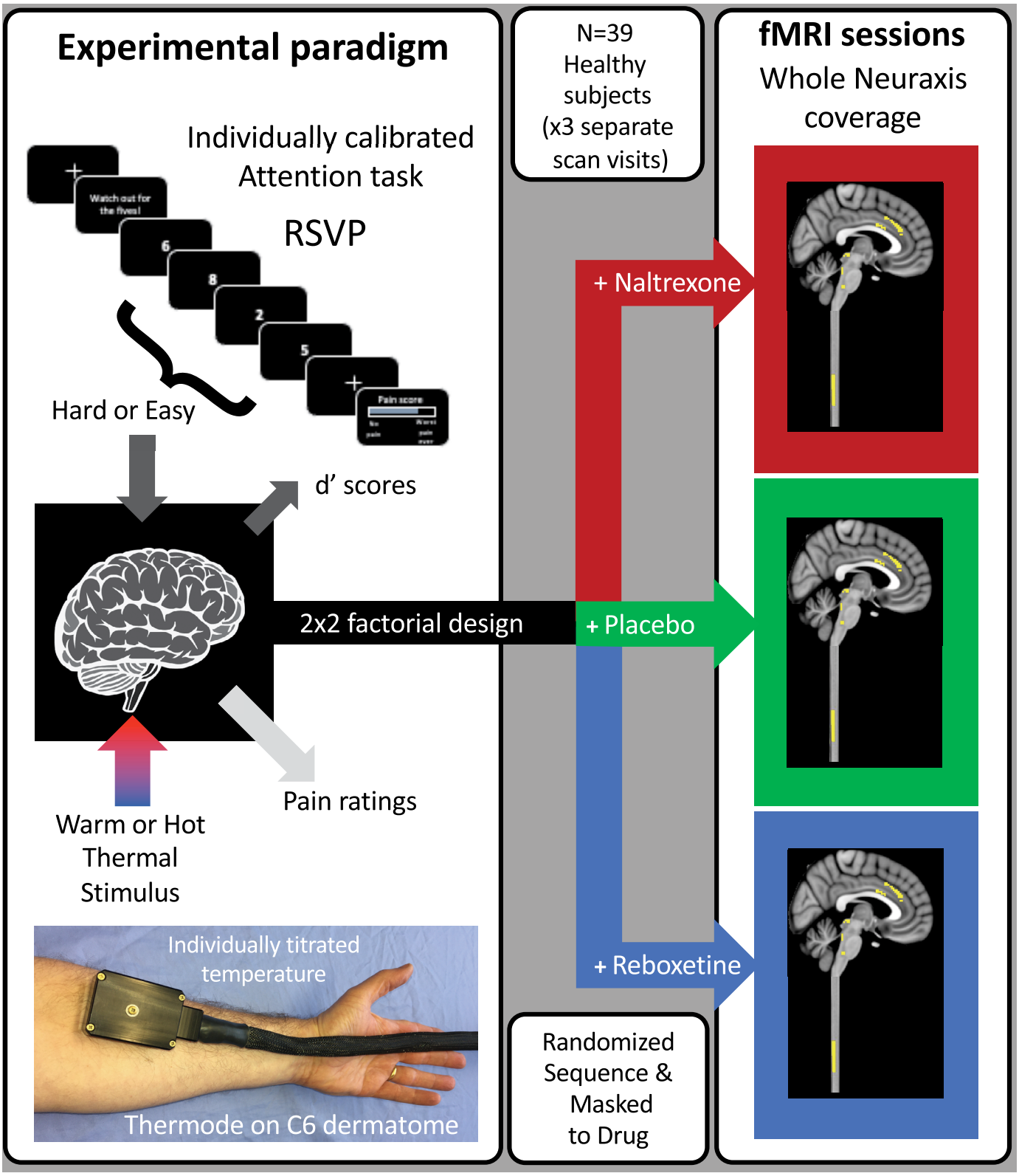

Figure 1. Experimental design. A total of 39 healthy subjects had thermal stimulation (to left forearm) while performing a rapid serial visual presentation (RSVP) task. The thermal stimuli were either warm or hot (individually titrated) and the task speed was adjusted for each subject to be either easy or hard (d' 70%, 16 blocks giving four repeats of each condition). This 2 × 2 factorial design allowed the interaction between task and temperature to be tested to identify the attentional analgesic effect. Each subject repeated the experiment on three separate days (at least 1 week apart) with a different drug on each occasion (naltrexone, reboxetine, or placebo) and had whole CNS fMRI.

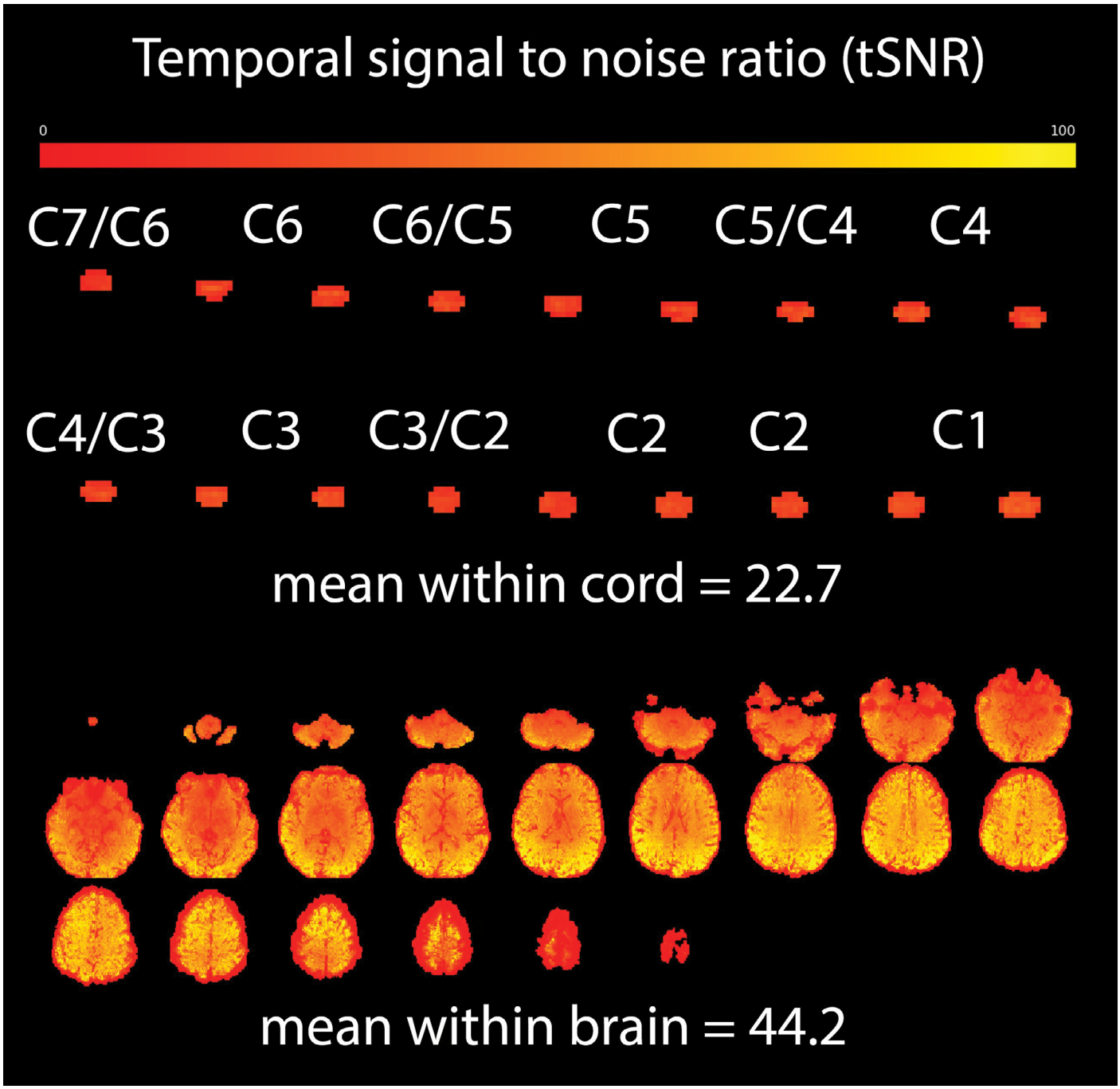

Figure 1—figure supplement 1. Representative temporal signal to noise ratio (tSNR) data for a single subject, acquired with identical parameters to those used in this study. Signal optimisation included manual selection of Z-shims, based on maximisation of cord signal and minimisation of distortion at each level in the cord (***Finsterbusch et al., 2012***). Image data (100 samples) acquired at rest were divided at the level of the odontoid process/dens, with that above (i.e. brain) motion corrected with a rigid body approach in FSL (6.0.3) and below (i.e. cord) with 2D correction in the Spinal Cord Toolbox (5.3.0), and the outputs generated with nearest neighbour interpolation to minimise smoothing. Following motion correction, the temporal mean was calculated and divided by the temporal standard deviation to produce the tSNR map.

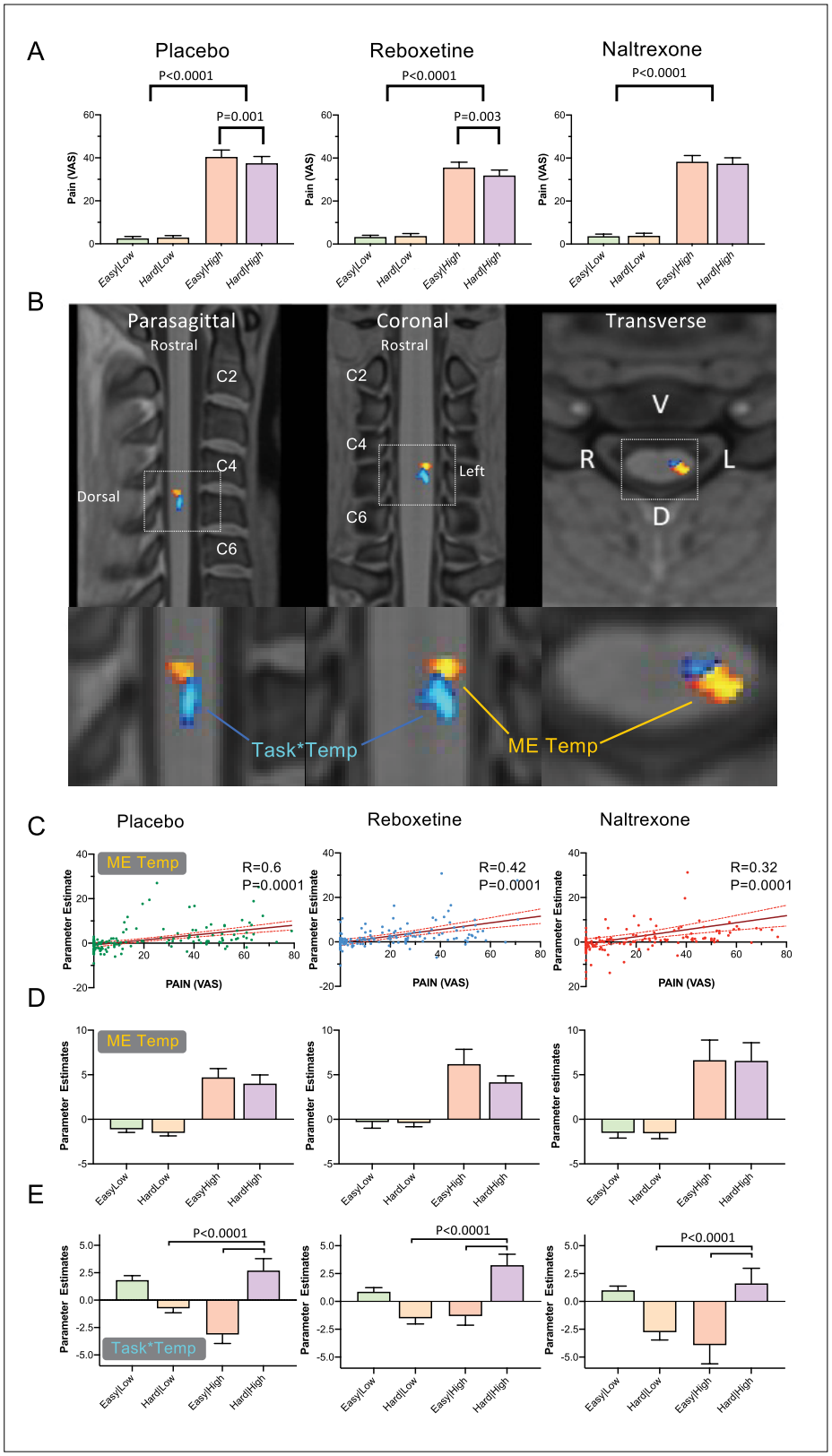

Figure 2. Main effect of temperature and task*temperature interaction in the spinal cord. (**A**) Pain scores across the four experimental conditions (i.e. easy|low, hard|low, easy|high and hard|high), for the three drugs. All conditions showed a main effect of temperature (two-way repeated measures ANOVA). Attentional analgesia was seen in the placebo and reboxetine limbs with a task*temperature interaction (F (1, 38) = 11.20, p = 0.0019 and F

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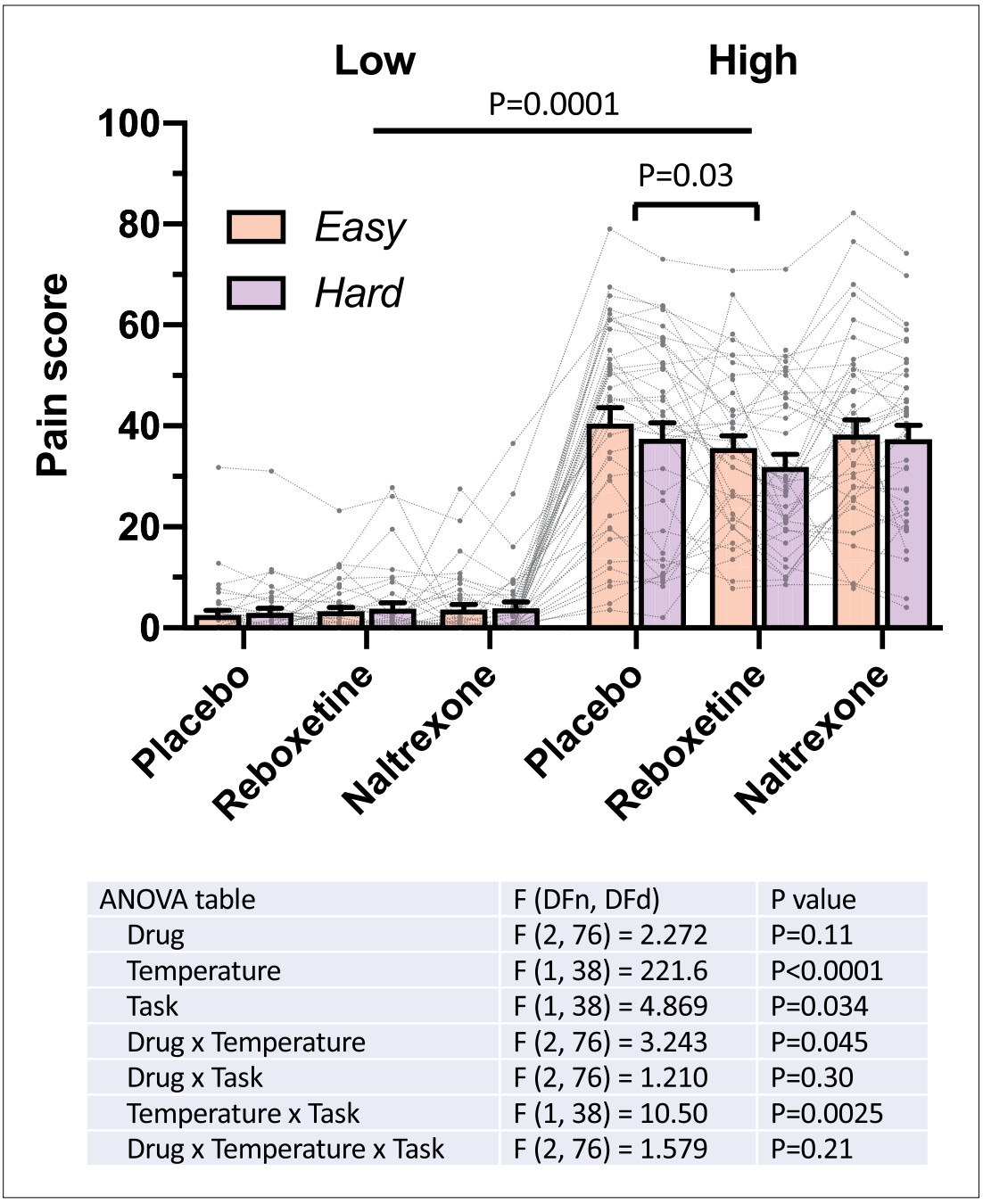
(1, 38) = 9.023, p = 0.004 respectively). In both cases, this was driven by lower pain scores in the hard|high versus easy|high condition (Sidak's post hoc test). In contrast, Naltrexone blocked the analgesic effect of attention as reflected in a loss of the task*temperature interaction (F (1, 38) = 0.4355, p = 0.5133). (**B**) Cervical spine fMRI revealed two distinct clusters of activity within the left side of the C6 cord segment. The first showing the main effect of temperature (red-yellow, $Spinal_{noci}$) and a second showing task*temperature interaction (blue-light blue, $Spinal_{int}$) (significance reported with $p < 0.05$ (TFCE) within a left sided C5/C6 anatomical mask). No cluster reached significance for the main effect of task. (**C**) Parameter estimates from the $Spinal_{noci}$ cluster showed a positive correlation with the pain scores across all conditions (Pearson's Correlation, 95% CI). (**D**) Parameter estimates from the $Spinal_{noci}$ cluster revealed a decrease in BOLD in the hard|high versus easy|high condition, seen in placebo and reboxetine arms but not in naltrexone. Note the similarity in pattern with the pain scores in (**A**). (**E**) Extraction of parameter estimates from the $Spinal_{int}$ cluster revealed an increase in BOLD in the hard|high condition, across all three drug sessions compared to the easy|high and hard||low conditions (Friedman test $p < 0.0001$). Mean ± SEM. Parameter estimates extracted from the peak voxel in each cluster.



ANOVA table	F (DFn, DFd)	P value
Drug	F (2, 76) = 2.272	P=0.11
Temperature	F (1, 38) = 221.6	P<0.0001
Task	F (1, 38) = 4.869	P=0.034
Drug x Temperature	F (2, 76) = 3.243	P=0.045
Drug x Task	F (2, 76) = 1.210	P=0.30
Temperature x Task	F (1, 38) = 10.50	P=0.0025
Drug x Temperature x Task	F (2, 76) = 1.579	P=0.21

Figure 2—figure supplement 1. Pain scores under the four experimental conditions (i.e. easy|low, hard|low, easy|high and hard|high), across the three drugs for each of the 39 subjects. A first level, three-way repeated measures ANOVA revealed the expected main effect of temperature ($F(1,38) = 221$, $p = 0.0001$), main effect of task ($F(1,38) = 4.9$, $p = 0.03$) and importantly a task*temperature interaction ($F(1, 38) = 10.5$, $p = 0.0025$). The first level analysis also showed a drug*temperature interaction on pain ratings ($F(2, 76) = 3.2$, $p = 0.04$). To further investigate the drug*temperature interaction, two second level three-way repeated measures ANOVAs were conducted for placebo vs reboxetine and placebo vs naltrexone (***Figure 2***). For reboxetine versus placebo, a drug*temperature interaction was revealed ($F(1, 38) = 5.060$, $p = 0.03$), with lower pain scores in high temperature condition in the reboxetine arm, indicating an analgesic effect of the drug. No drug*temperature interactions were observed in the ANOVA contrasting naltrexone with placebo. Mean + SEM with individual participants data.

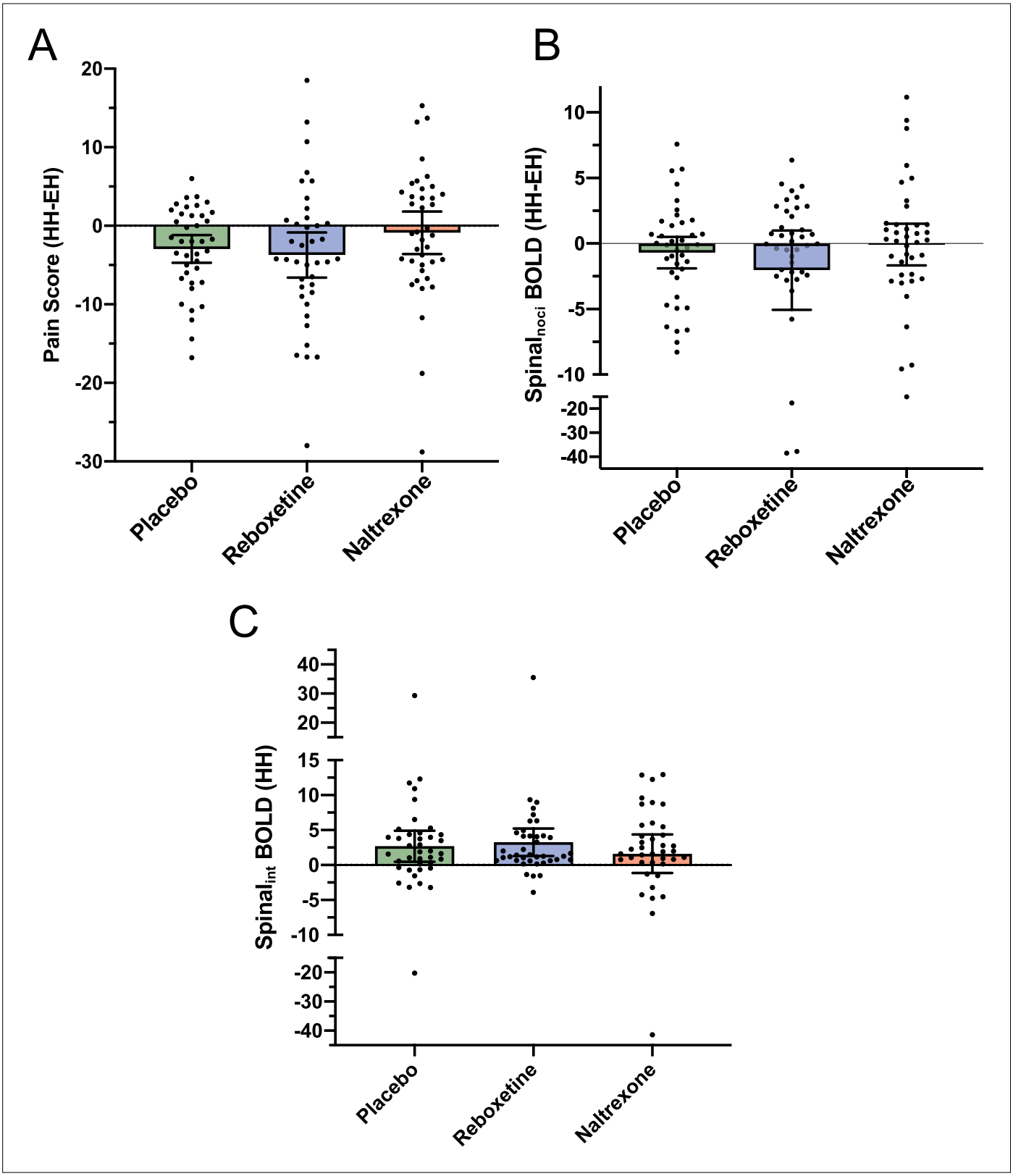


Figure 2—figure supplement 2. Influence of drug on attentional analgesia and on spinal BOLD parameter estimates. (**A**) Attentional analgesia effect reflected as the difference in pain score between the easy and hard condition in the high temperature condition (mean ± 95% confidence interval). The placebo and reboxetine groups show a significant reduction in pain scores in the high hard condition ie attentional analgesia ($p = 0.0016$ and $p = 0.013$, respectively) whereas there is no significant effect of naltrexone ($p = 0.51$, one sample t-tests). The corresponding effect sizes (Cohen's Dz)

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are Placebo –0.55, Reboxetine –0.42 vs Naltrexone –0.11. The confidence interval for naltrexone spans zero and equivalence testing showed that the magnitude of the effect was smaller than a 6% (2.3 point) reduction in pain score ($p = 0.049$, using the TOST approach ***Lakens, 2017***) and less than the analgesic effect seen in the presence of reboxetine or placebo. (**B**) Extraction of the BOLD parameter estimates from the $Spinal_{noci}$ cluster for the HH-EH conditions showed a similar pattern of means but with an increased dispersion of values (note the break in the y-axis scale) reflecting the signal to noise associated with spinal cord functional imaging. As a consequence, the 95% confidence intervals all cross zero and there are no significant differences between the groups. (**C**) Extraction of the BOLD parameter estimates from the $Spinal_{int}$ cluster for the High Hard condition showed that the group means were significantly increased in the placebo ($p = 0.018$) and reboxetine ($p = 0.0018$) conditions but not in the presence of naltrexone ($p = 0.24$). (Mean ± 95% CI, one sample t-tests).

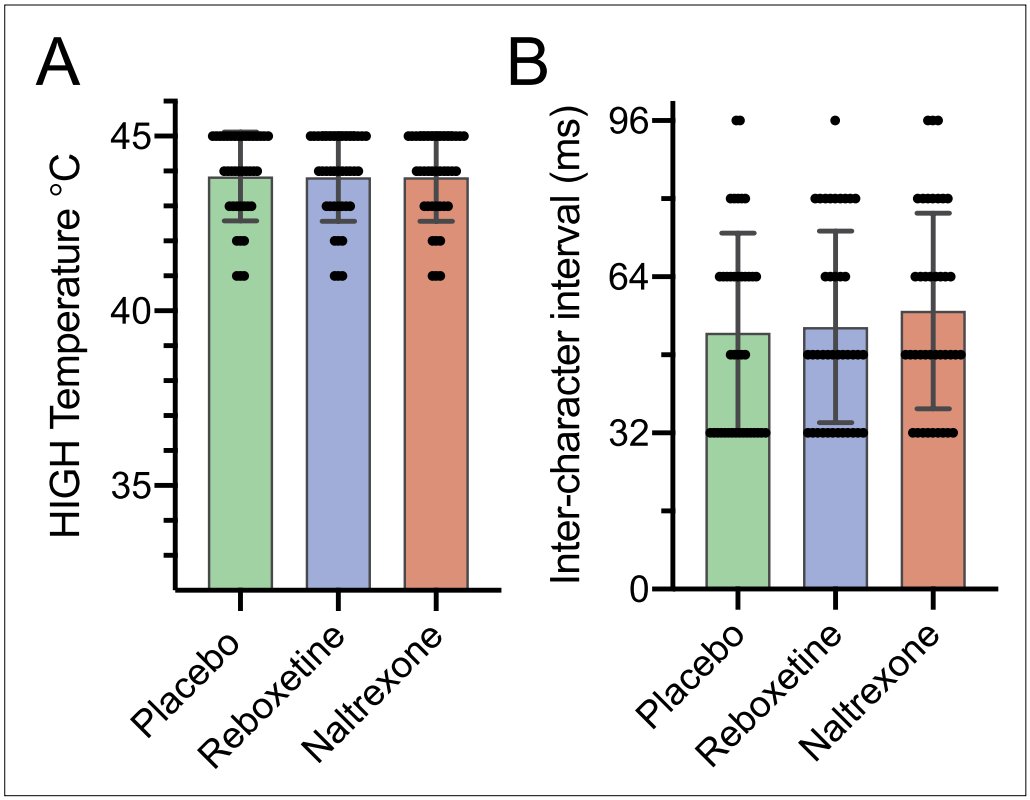

Figure 2—figure supplement 3. Temperature delivered and task speed across the three drug conditions. (**A**) Administration of Reboxetine or Naltrexone did not change the individually calibrated HIGH thermal stimulus required to evoke a 6/10 pain score (Mean ± SD). (**B**) Similarly, drug administration had no effect on RSVP task speed as reflected in the inter-character presentation interval (Mean ± SD, Friedman tests NS).

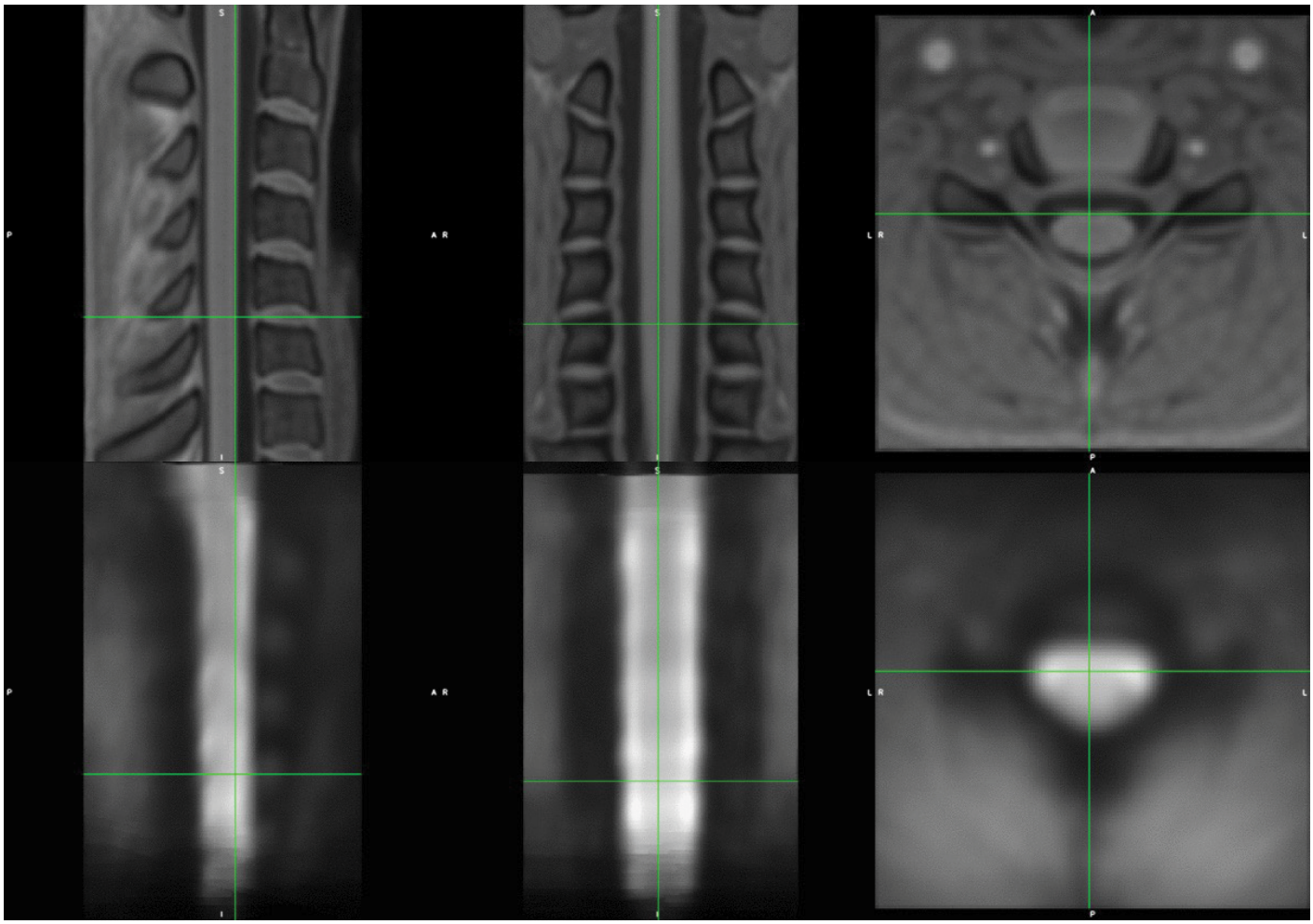

Figure 2—figure supplement 4. Analysis of pooled data for main effects and interaction within the cord. Top: PAM50 template T1-weighted cervical cord, bottom: mean functional image from all 39 subjects acquired during the placebo condition, shown following non-linear registration to the template. Note the good agreement with intervertebral disc levels and ventral surface of the cord. The registration pipeline included two steps: (1) registration of subject's own T1-weighted structural scan to PAM50 T1-weighted template and (2) registration of acquired functional images to PAM50 template (T2*-weighted) to using the output from step one as an initial warping. This last step assumed that the subject's T1-weighted scan and EPI data were in reasonable agreement, which was confirmed by visual inspection. Note that in every case it was found that manual intervention was required to improve the cord mask for the functional images.

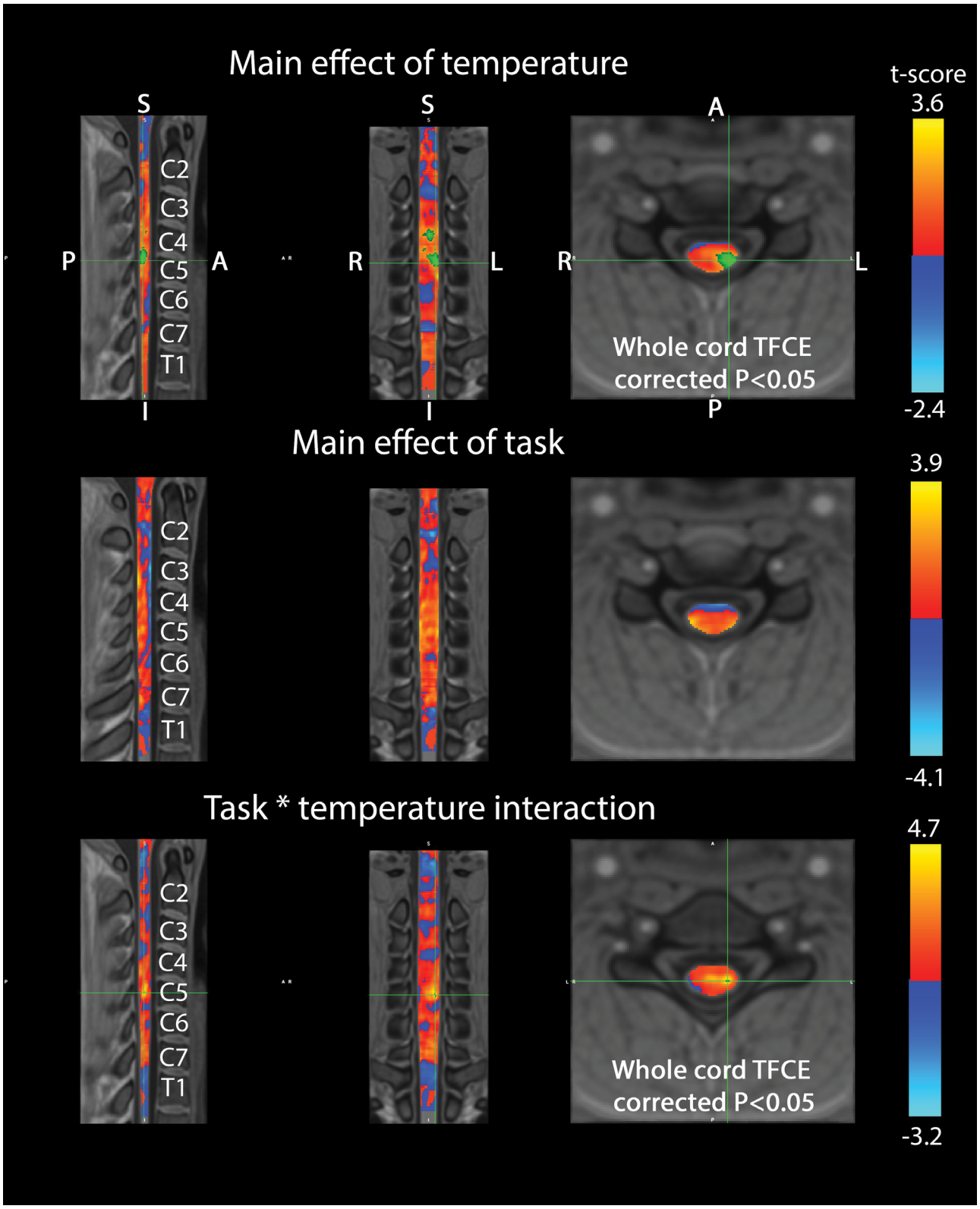

Figure 2—figure supplement 5. Analysis of pooled data for main effects and interaction within the cord. Inference was performed without masking for a specific vertebral level and produced t-scores shown in Red-Yellow (positive) and Blue-Light blue (negative). Importantly, the unmasked analysis confirmed the presence of a main effect of temperature at the C5/C6 level within the left dorsal horn region (shown in Green, with cross-hair on voxel of with lowest p-value), with TFCE corrected $p < 0.05$. Similarly, unmasked analysis provided confirmatory evidence for the existence of a task

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x temperature interaction located within the left dorsal horn region at the C5/C6 level (Green, cross-hair on voxel with lowest p-value), with TFCE corrected $p < 0.05$. No main effect of task was observed within the cord, in agreement with masked analysis.

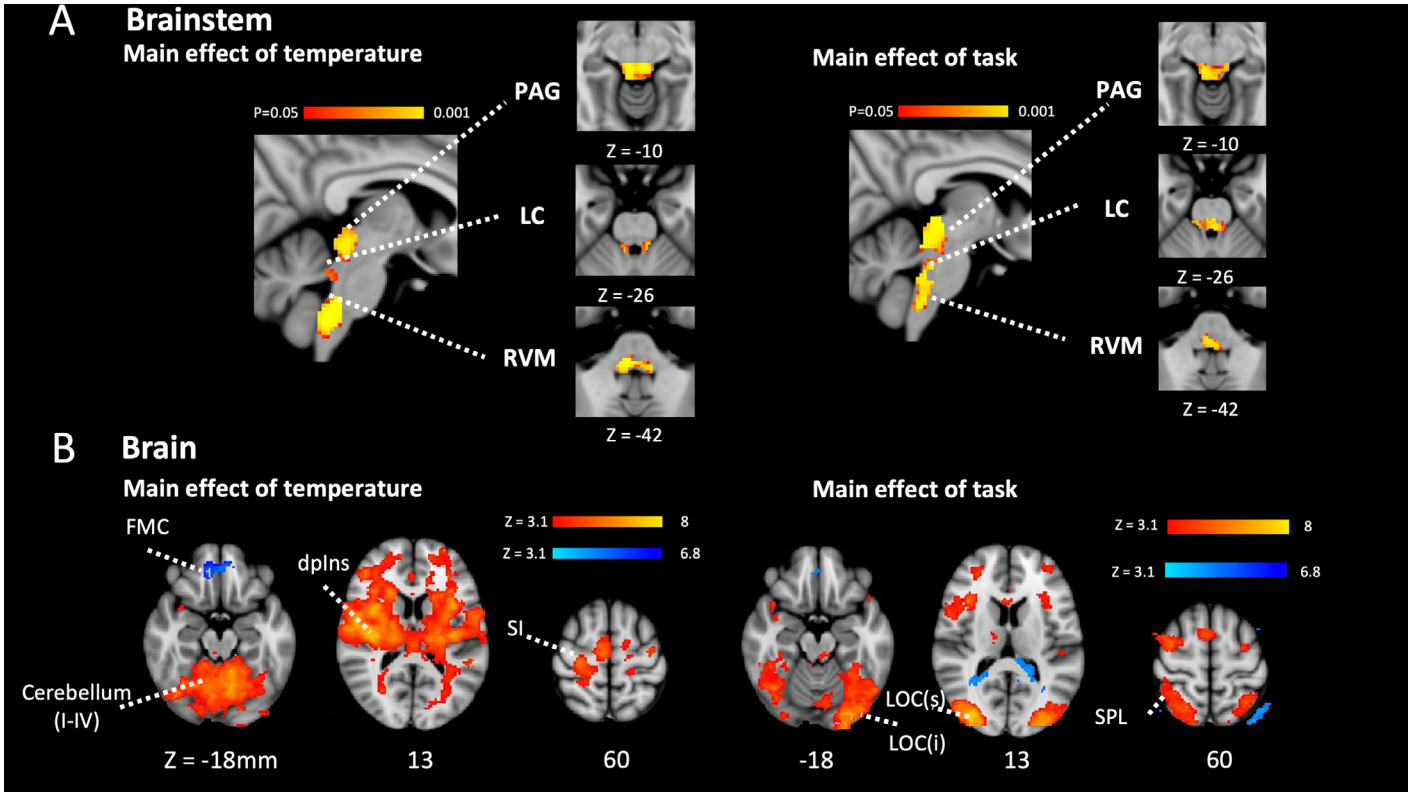

Figure 3. Main effect of task and temperature in Brainstem and Cerebrum. (**A**) Main effect of temperature and task in the brainstem after permutation testing with a whole brainstem mask showing clusters of activation in PAG, bilateral LC and RVM. Activity reported with corrected p< 0.05 (TFCE). (**B**) Main effects of temperature and task in brain. In the main effect of temperature contrast there were clusters of activation in a number of pain related sites including in the contralateral primary somatosensory cortex, the dorsal posterior insula and the PAG (red-yellow). The frontal medial cortex de-activated (blue-light blue). In the main effect of task contrast there were clusters of activation in the visual and attention networks including superior parietal cortex, the frontal pole, and the anterior cingulate cortex (red-yellow). The posterior cingulate cortex and lateral occipital cortex showed de-activation (blue-light blue). Activity was estimated with a cluster forming threshold of Z > 3.1 and FWE corrected p < 0.05. (PAG – Periaqueductal grey, LC – Locus coeruleus, RVM – Rostral ventromedial medulla, FMC – Frontomedial cortex, dpIns – dorsal posterior insula, SI – primary somatosensory cortex, LOC – Lateral ocipital cortex (sup and inf), SPL Superior parietal lobule.).

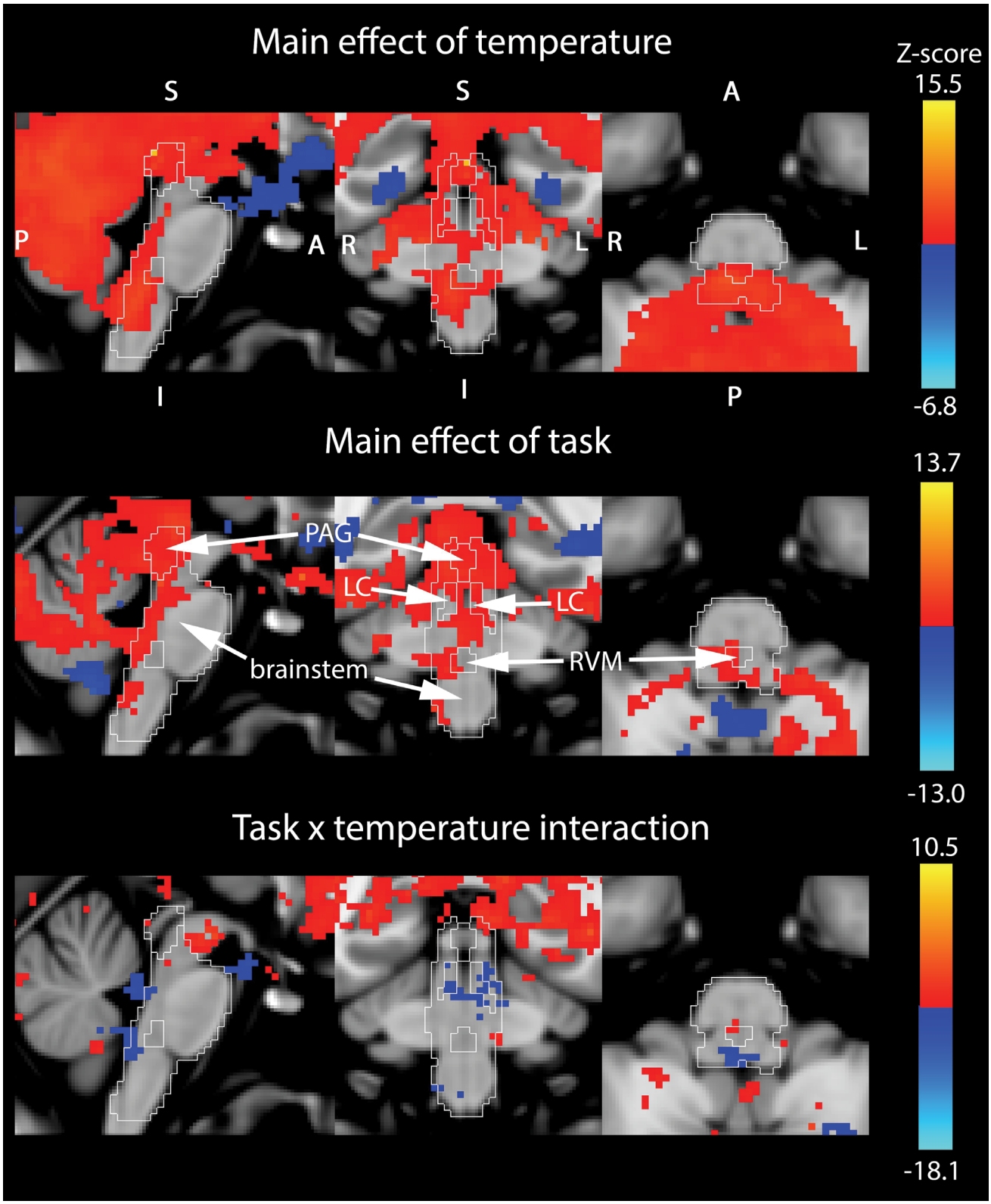

Figure 3—figure supplement 1. Whole brain mixed effects analysis of pooled data (inputs are the average of each subject's three sessions) for the three contrasts (main effects of temperature, task and their interaction). Slices shown (left to right) **(i)** midline sagittal, (ii) coronal through the PAG, bilateral LC and RVM masks, and (iii) axial at the level of the midline RVM mask. To allow visualisation of underlying anatomy, data were thresholded at an *uncorrected* p-value of 0.05 (i.e. Z > 1.65). The location of relevant masks are outlined in white, with labels shown. Also included is the brainstem

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mask derived from the Harvard-Oxford sub-cortical probabilistic atlas, which was thresholded at 50% and used for estimating brainstem activity reported in the manuscript (rather than the whole brain analysis shown here). Assignment of activity to specific nuclei was based on overlap with probabilistic brainstem nuclei masks (***Brooks et al., 2017***). Positive Z-scores are shown in Red-Yellow colours, whilst negative ones are in Blue-Light blue. Activity was rarely observed in the 4th ventricle, nor in the aqueduct, indicating that physiological noise was adequately corrected for with the chosen scheme (see ***Brooks et al., 2008***; ***Kong et al., 2012*** for more details).

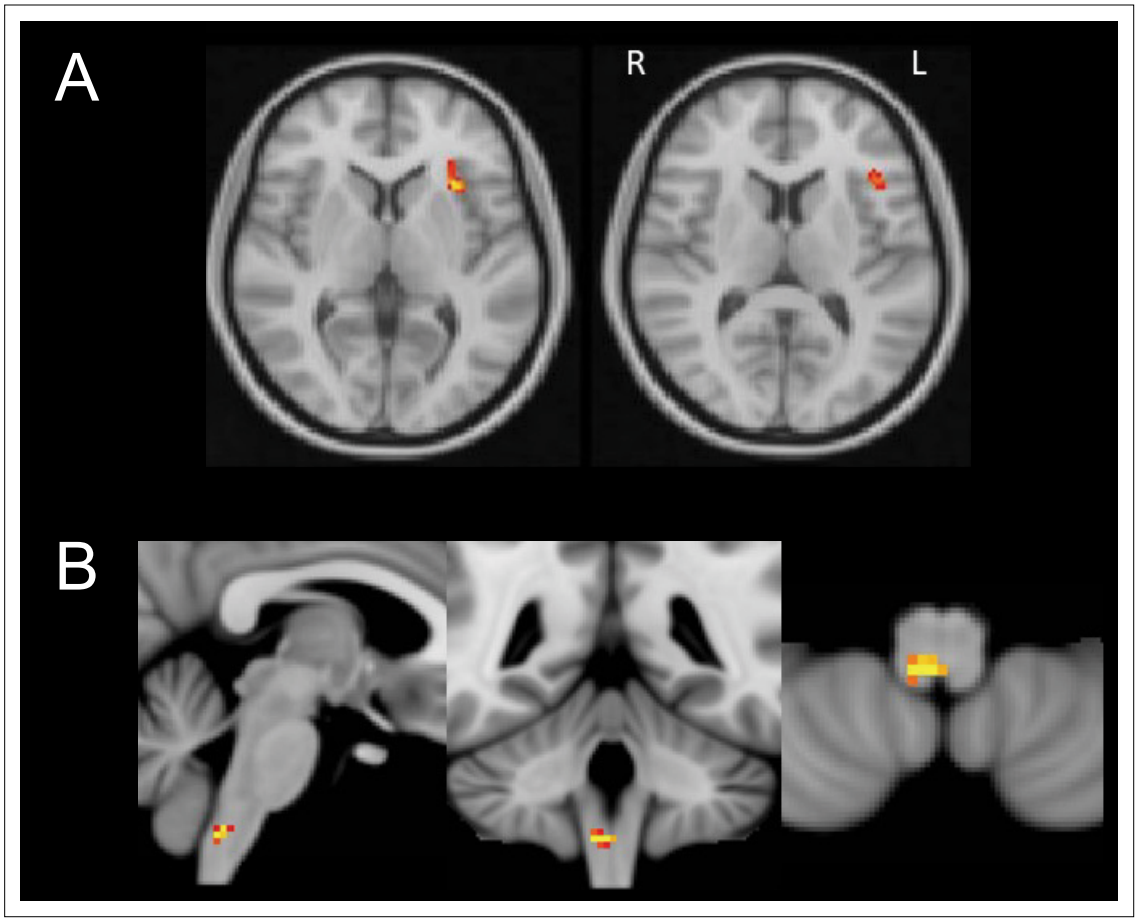

Figure 3—figure supplement 2. Anterior Insula and medulla response after Naltrexone administration. (**A**) The anterior insula responded more strongly in the naltrexone than in the placebo in the main effect of task (obtained with permutation testing with a main effect of task mask, obtained from the pooled analysis). (**B**) A cluster in the lower medulla responded more strongly in the naltrexone than in the placebo main effect of temperature. Result obtained with permutation testing (using a main effect of temperature brainstem mask, obtained from the pooled analysis). TFCE corrected $p < 0.05$.

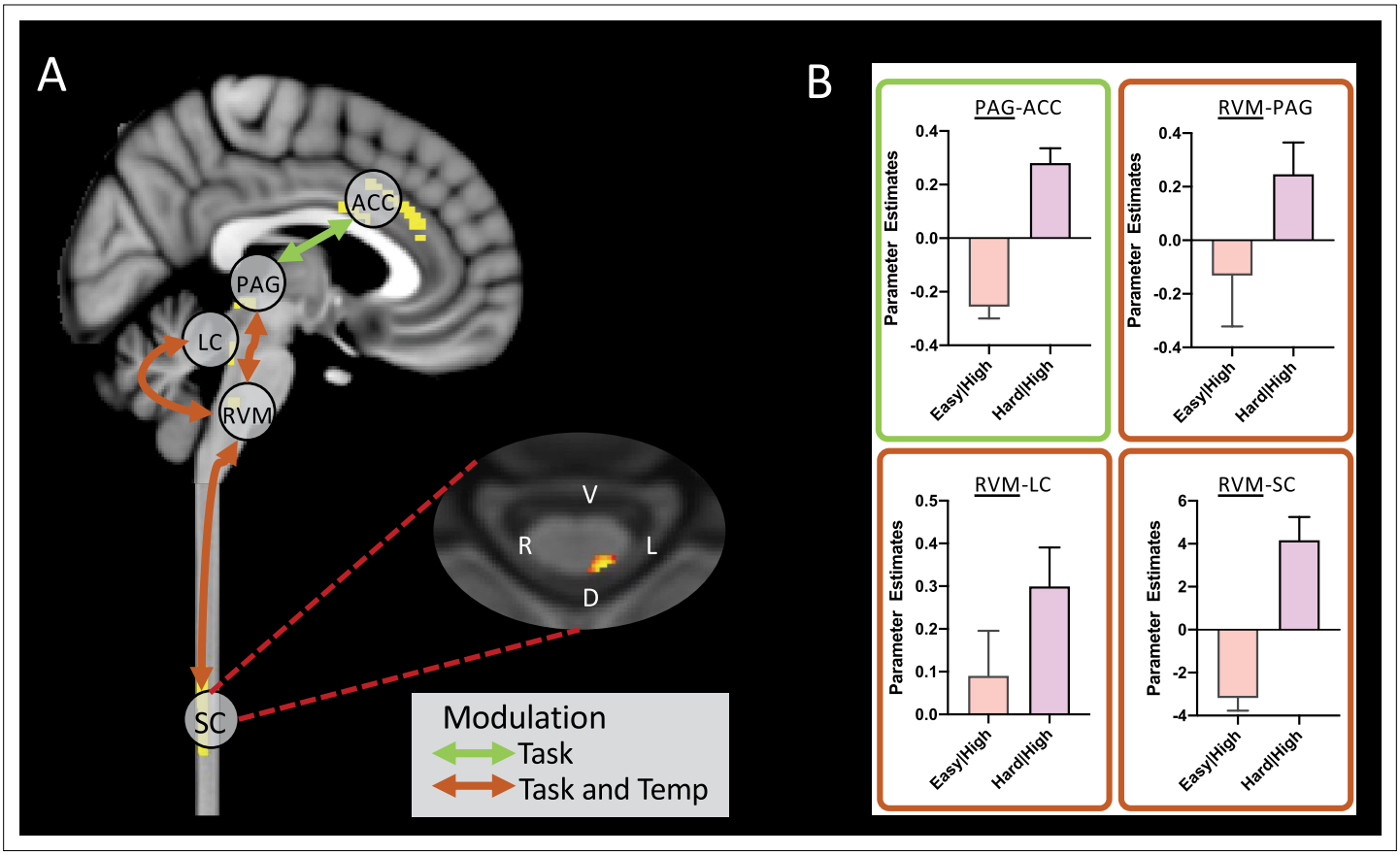

Figure 4. Summary of significant connection changes revealed by the gPPI analysis (placebo condition only). (**A**) Permutation testing revealed a significant change in connectivity in the main effect of task contrast between ACC and PAG, and in the task*temperature interaction contrast between PAG and RVM, LC and RVM, and importantly RVM and spinal cord. Masks used for time-series extraction are shown in the sagittal slice (yellow). The spinal cord axial slice shows the voxels with significantly connections with RVM (threshold at = 0.1 for visualisation purposes). (**B**) Extraction of parameter estimates revealed an increase in coupling in the analgesic condition for all of these connections (i.e. hard|high). (Mean ± SEM).

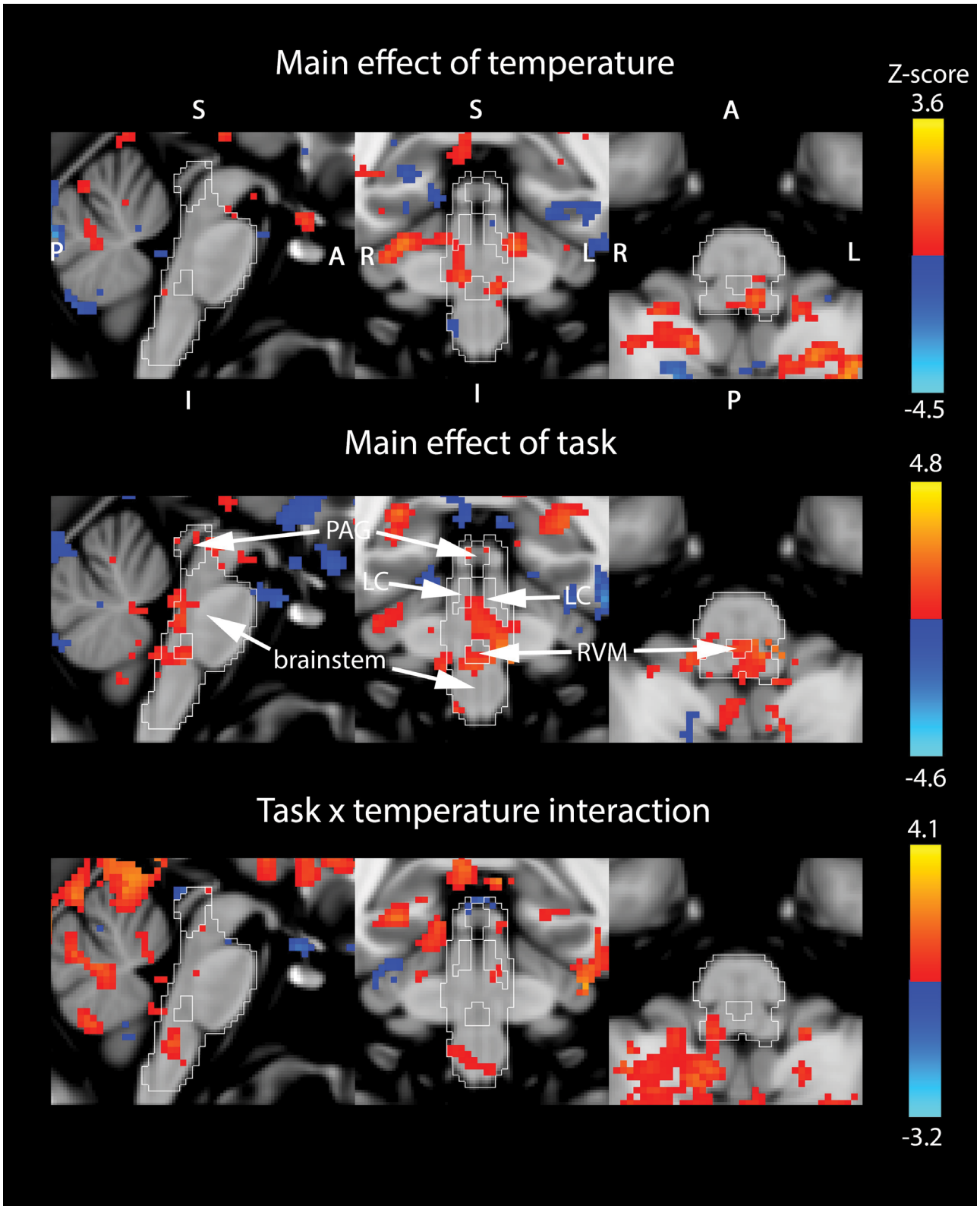

Figure 4—figure supplement 1. Unmasked whole brain group data for effective connectivity analysis of the placebo condition only. For each subject, the seed was extracted for the main effect of temperature (within the pooled simple main effects data) within the RVM. That is, a functional mask was derived from the group data, masked anatomically then applied to each subject separately to identify their peak voxel time series (the seed). Subsequently, the connectivity profile was estimated for each subject using generalised psychophysiological analysis (gPPI), with separate contrasts

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between the gPPI regressors for the three conditions (main effects of task, temperature and their interaction). To allow visualisation of underlying anatomy, these whole brain data were thresholded at an uncorrected p-value of 0.05 (i.e. $Z > 1.65$). The location of relevant masks are outlined in white (see labels on previous brainstem figure). Positive Z-scores are shown in Red-Yellow colours, whilst negative ones are in Blue-Light blue.

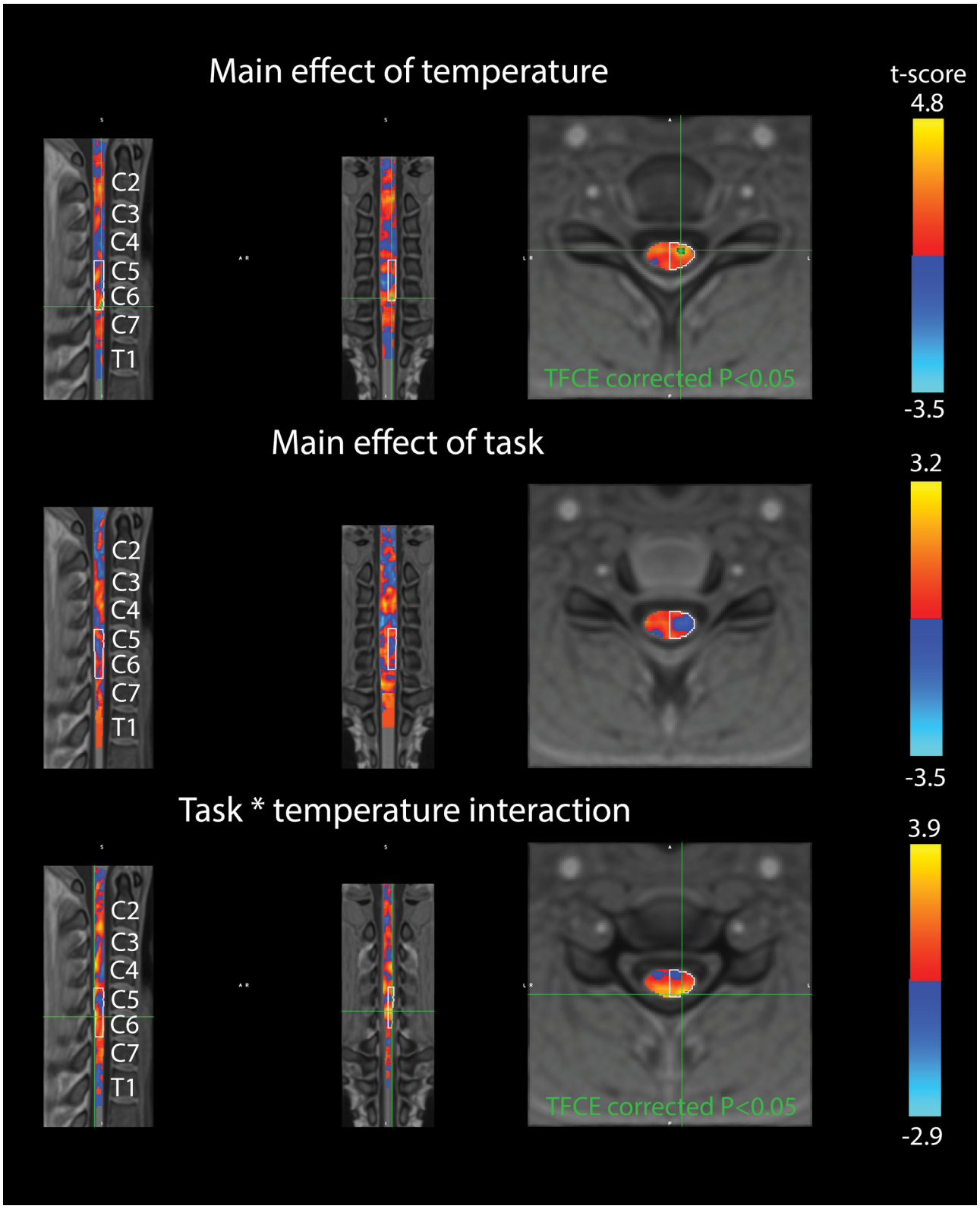

Figure 4—figure supplement 2. Unmasked group cord data from connectivity analysis of the placebo condition shown on the PAM50 spinal cord template. For each subject, the physiological regressor was extracted from a functional mask representing the main effect of temperature within the RVM for the placebo condition. Subsequently, generalised psychophysiological interaction (gPPI) regressors were formed for each of the conditions and contrasts between them created. The data represent uncorrected positive (Red-Yellow) and negative (Blue-Lightblue) t-scores, which are the output from

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RANDOMISE. Vertebral levels are indicated on sagittal section (left side of image). Due to masking steps in the registration pipeline it was not possible to include tissues outside the cord. To aid interpretation of the patterns of activity, the left C5-C6 vertebral mask is shown (white outline). Significant group activity detected within the mask for each contrast are shown in green, with TFCE corrected $p < 0.05$.

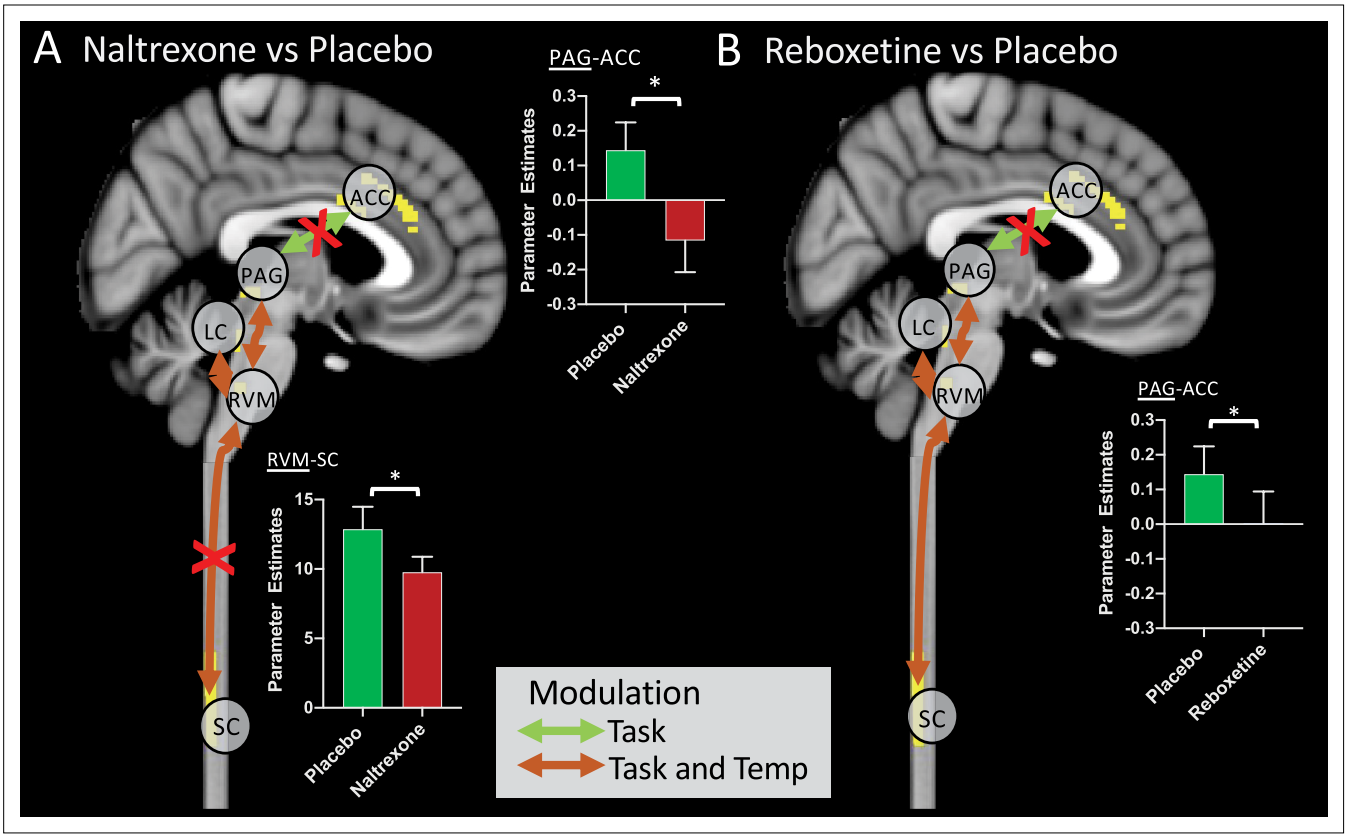

Figure 5. Alteration of functional connectivity after dosing with naltrexone or reboxetine compared to placebo. The ACC-PAG connection was significantly weakened by Naltrexone and Reboxetine administration. The RVM-spinal cord connection was significantly weakened by Naltrexone. Red crosses indicate significantly weaker connections after drug. Inset bar plots show BOLD parameter estimates extracted from the PAG-ACC and RVM-spinal cord connections. (Means ± SEM, paired t-test, *p < 0.05).