Supplementary Information

Mogil et al.

Pain sensitivity and vasopressin analgesia are mediated by a gene-sex-environment interaction.

Figure S1. Positional refinement of the formalin test QTL, *Nociq2*, using RC strains and marker-assisted subcongenic lines derived from them (see Online Methods and **Fig. S2**). The *x*-axis of graph **A** shows the genotype of the various strains on mouse chromosome 10 (in Mb). A/J alleles are shown in green; B6 alleles are shown in red (note that all strains shown are homozygous at all loci). The pie charts to the left of the strain names illustrate the percentage of total genome from each founder strain (13.25% for AcB and BcA RC strains; <1% for subcongenic strains 64SCA and 64SCB). Blending of colors indicates uncertainty as to the precise crossover location. Phenotypic values represent the mean % positive samples in the late-phase formalin test (± SEM in parentheses); *n*=12–50 mice/strain. Colored squares next to the phenotypic values indicate statistical equivalence (Dunnett's one-sided posthoc test) to the A/J (green) or B6 (red) phenotype. The phenotypic data from the seven RC strains are consistent with the responsible polymorphism being located distal to *D10Mit25* but proximal to *D10Mit103* (i.e., in the purple box shown). The subcongenic lines also support that location, and the overwhelmingly B6-derived genotype of these lines rules out the responsible gene lying elsewhere on the genome. The *x*-axis of graph **B** shows the genotype of informative strains at individual microsatellites (arrows; all *D10Mit*xxx) located at the distal end of chromosome 10 (113–126 Mb).

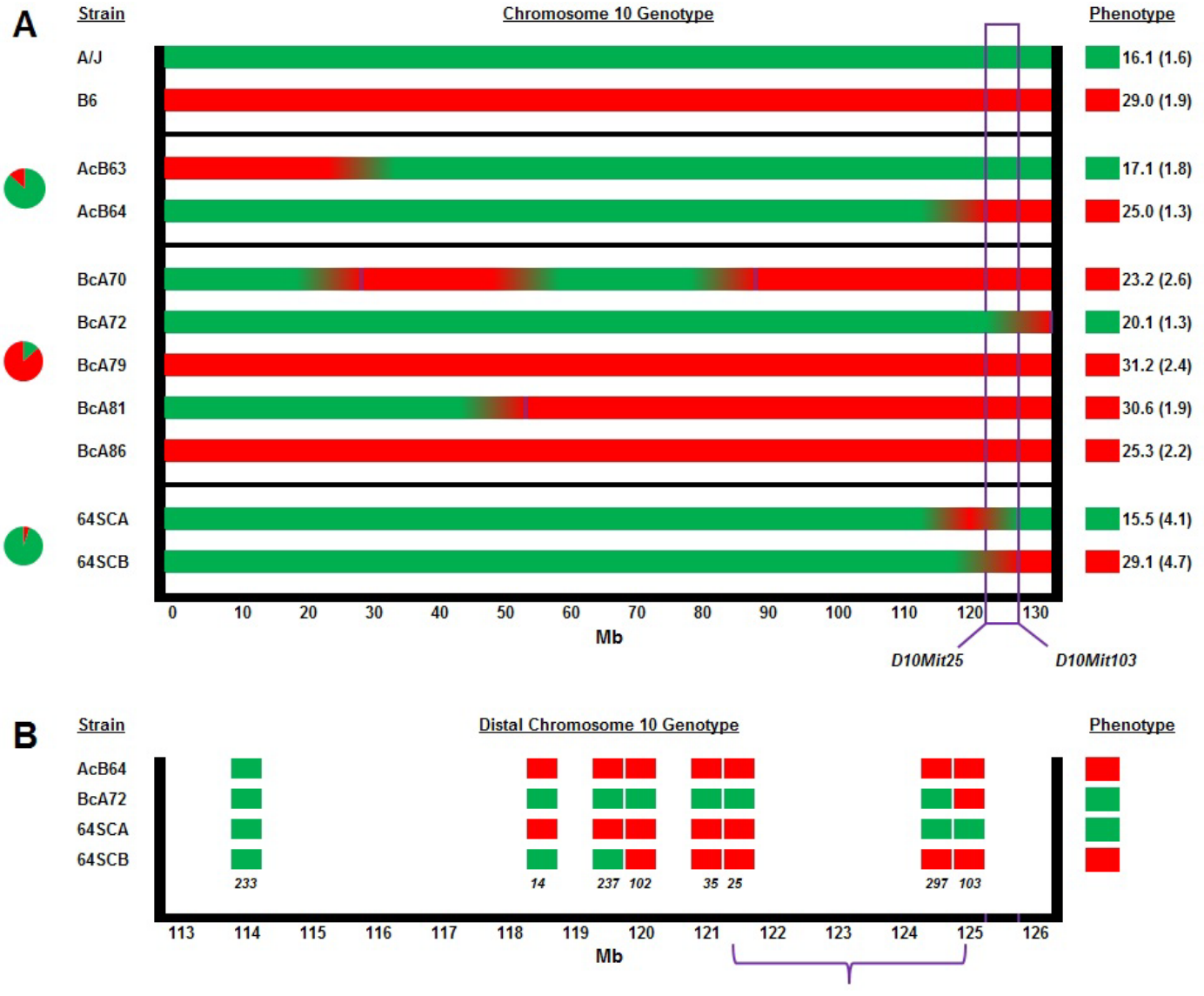

Figure S2. Sensitivity of marker-assisted ("speed") congenic (and associated wildtype) lines derived from RC strains AcB64 (**A**-**C**) and BcA72 (**D**-**F**) on the 5% formalin test. AcB64 mice were backcrossed to A/J for five generations to eliminate all B6 genome except for that on distal mouse chromosome 10 (in congenic lines only); four wildtype lines (64WT1–64WT4) and four congenic lines (64C1–64C4) resulted. Similarly, BcA72 mice were backcrossed to B6 for five generations to eliminate all A/J genome except for that on distal mouse chromosome 10 (in congenic lines only); only one wildtype line (72WT2) and three congenic lines (72C1, 72C2, 72C4) survived. Early phase (F_{early}; 0-10 min postinjection) results are shown in **A**,**D**; late phase (F_{late}; 10-60 min postinjection) results are shown in **B**,**E**; edema at 60 min (expressed as the difference between injected and uninjected hindpaw weight as a percentage of body weight) is shown in **C**,**F**. Bars represent means ± S.E.M.; n=12–25 mice/genotype. With no reason to distinguish among individual lines, we performed t-tests on congenic versus wildtype lines in aggregate. *p<0.05; ***p<0.001. As can be seen, AcB64-derived congenic lines displayed significantly *higher* sensitivity on the formalin test in both F_{early} and F_{late}, with no differences in edema. BcA72-derived congenic lines displayed significantly *lower* sensitivity on the formalin test in F_{late}, but not F_{early}, with no differences in edema.

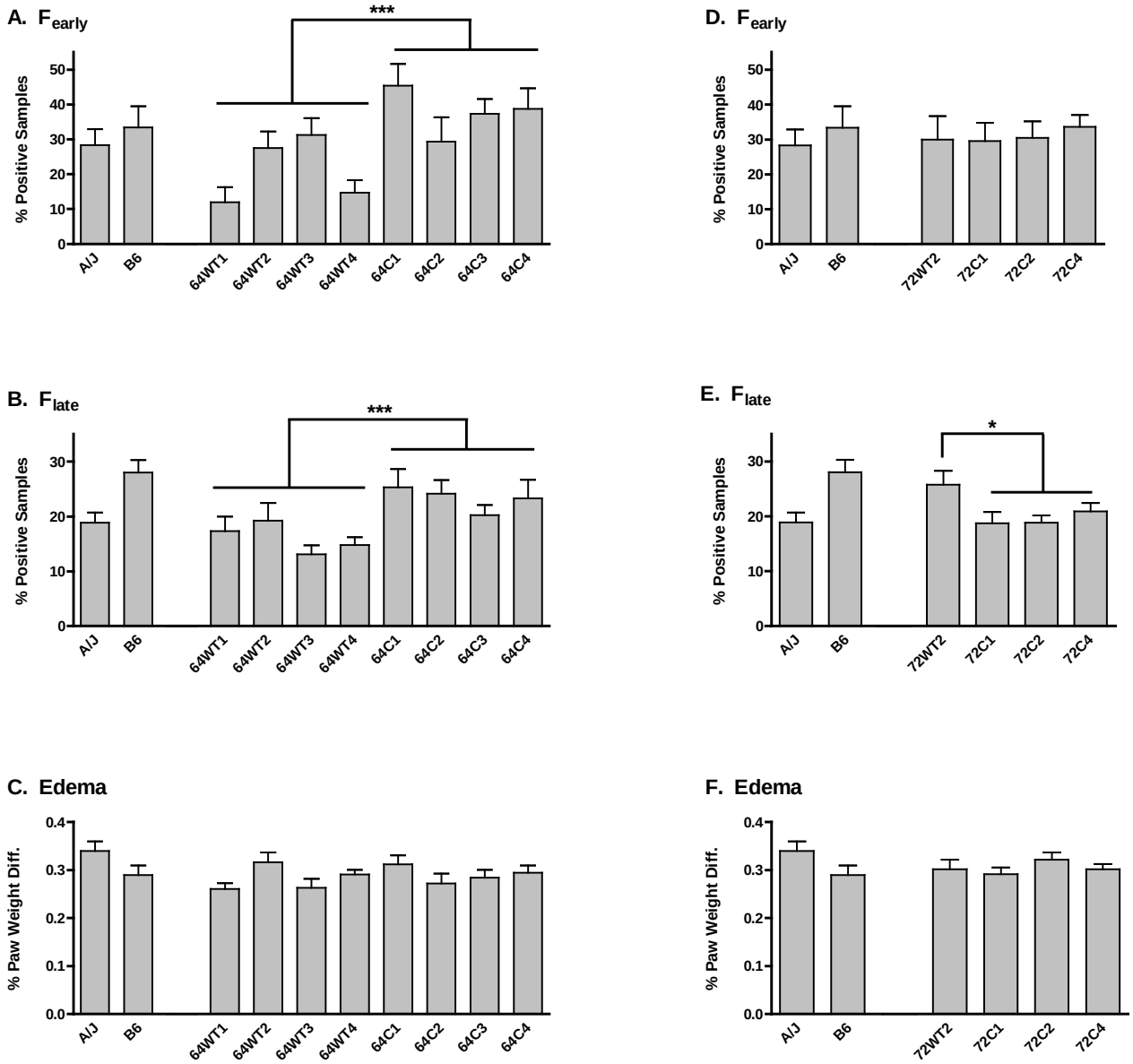

Figure S3. Concentration-response relationships of A/J and B6 mice, and interval-specific subcongenic lines 64SCA and 64SCB, on the formalin test. For construction of subcongenic lines see Online Methods; both have homozygous A/J alleles throughout the genome except for the distal-most portion of mouse chromosome 10, where 64SCA possesses A/J alleles up until 121.7–124.4 Mb and B6 alleles thereafter, and where 64SCB possesses B6 alleles from 120.3–124.4 Mb and A/J alleles thereafter. Early phase (F_{early}; 0-10 min postinjection) results are shown in **A**; late phase (F_{late}; 10-60 min postinjection) results are shown in **B**; edema at 60 min (expressed as the difference between injected and uninjected hindpaw weight as a percentage of body weight) is shown in **C**. Symbols/bars represent mean ± S.E.M.; n=5–13 mice/genotype/concentration. A two-way ANOVA (genotype x formalin concentration) performed on F_{early} data revealed a significant main effect of genotype ($F_{3,101}$=4.8, p<0.005) only, whereas the same ANOVA performed on F_{late} data revealed significant main effects of genotype ($F_{3,101}$=14.1, p<0.001), formalin concentration ($F_{3,101}$ = 7.0, p<0.001), and a genotype x concentration interaction ($F_{9,101}$=2.3, p<0.05). As can be seen, B6 and 64SCB mice displayed dose-dependent increases in F_{late} licking/biting behavior, whereas A/J and 64SCA mice displayed low levels of behavior at all formalin concentrations. ANOVAs performed on edema data also revealed significant main effects and a significant interaction (all p's<0.05), largely driven by variable edema at low formalin concentrations. At higher concentrations (2.5-5%), however, where genotype differences in behavioral responses were most notable, levels of edema were similar across genotype.

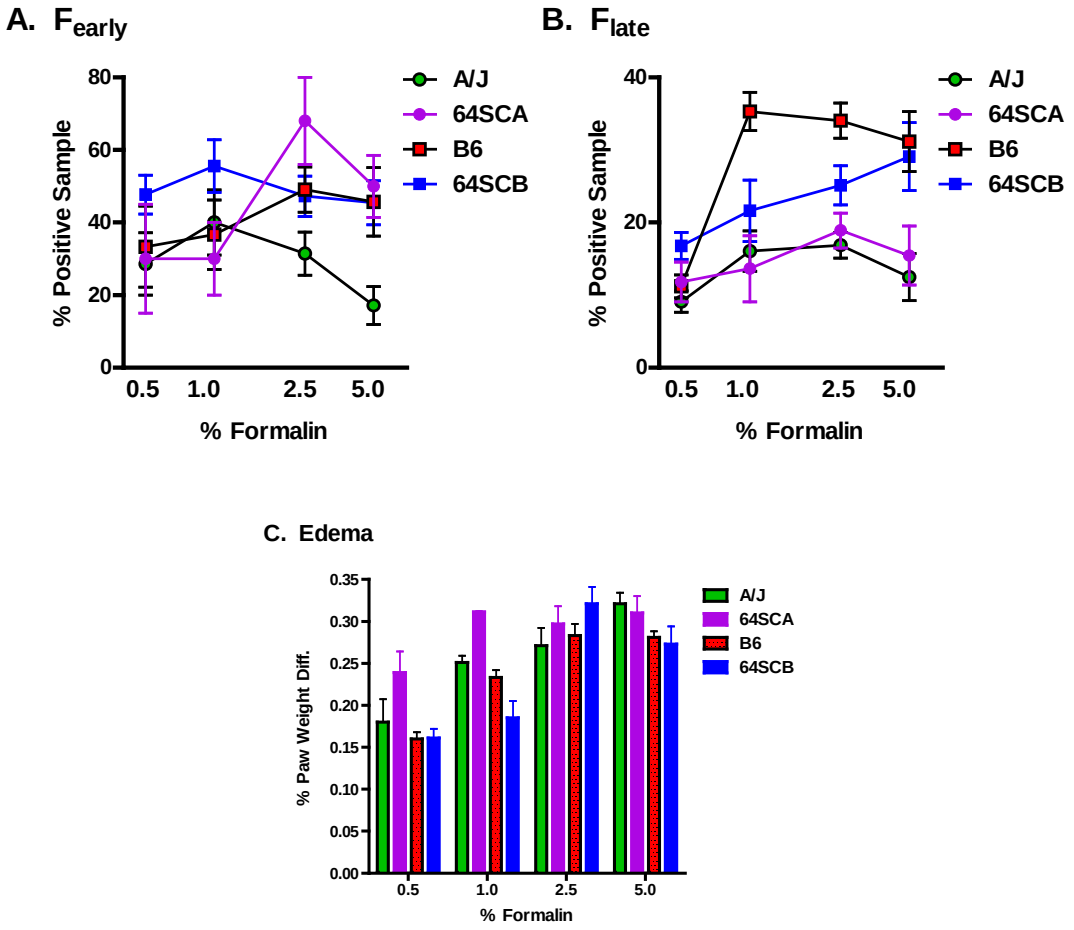

Figure S4. Racial differences in rs10877969 allele frequencies and their effect on capsaicin pain. In **A**, genotype and allele frequencies are presented; a χ^2 test revealed highly significant differences in allele frequencies in African-American compared to Caucasian subjects. The race difference is also present in HapMap data for this SNP. In graphs **B** and **C**, the effect of rs10877969 genotype on capsaicin pain is presented by racial group for male (**B**) and female (**C**) subjects. Bars represent mean ± S.E.M. capsaicin VAS score. The race x sex x genotype interaction approached significance ($F_{2,89}$ = 1.9, p=0.15). Importantly, the same sex x genotype x stress interaction shown in **Fig. 3** of the main text is observed whether or not African-Americans are included or excluded from analysis, so it is highly unlikely our main results are confounded by population admixture.

A

Racial Group	GG	GA	AA	G allele	A allele	p^a
Caucasian	2	13	38	0.28	0.72	
African-American	9	14	12	0.66	0.34	0.0005
Asian	1	5	8	0.43	0.57	0.30

[a]Allele frequencies compared to Caucasian by χ^2 test.

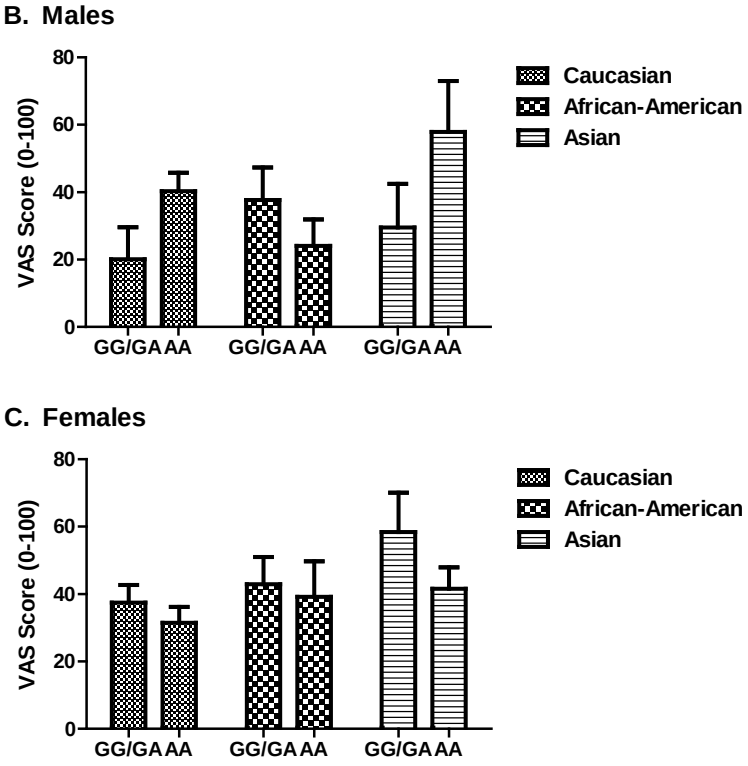

Figure S5. Effect of rs10877969 genotype on a "stress-induced analgesia" factor uncovered using factor analysis of gain constants for phasic, tonic and integrator components describing subjects' dynamic pain responses to capsaicin. The repeated ratings of capsaicin pain intensity of the subset of subjects' of the capsaicin pain association study data set with AVPR1A genotype, VAS and stress ratings (n=80) were analyzed as described previously (see Balaban et al., *J. Pain*, 2005; Lariviere et al., *J. Pain*, 2005) to estimate tonic, phasic, and integrator gain constants with custom MATLAB (Mathworks) programs.

A factor analysis was performed on rs10877969 genotype, mean VAS, stress ratings, and square root transformed tonic, phasic and integrator gain. The equamax rotation was used as a compromise of varimax and quartimax criteria. These factor scores were calculated for each subject (based upon z-scores of the original dependent variables). The factor loadings plot is shown in A. The three factors explained 78% of the total variance (Factor 1: 34.6%; Factor 2: 22.6%; Factor 3: 20.5%). Of particular interest is Factor 3, which appears to reflect stress-induced analgesia: the difference between stress and mean VAS. Bars in B show least squares means ± SEM for Factor 3 scores calculated for each subject (based upon z-scores of the original dependent variables). Factor 3 showed a predominant influence of rs10877969 (B), which produced a highly significant main effect ($F_{1,76}$ = 7.429, $p<0.01$). The AG/GG males had greater Factor 3 scores than either the AA male ($p<0.01$) or AA female groups ($p<0.05$).

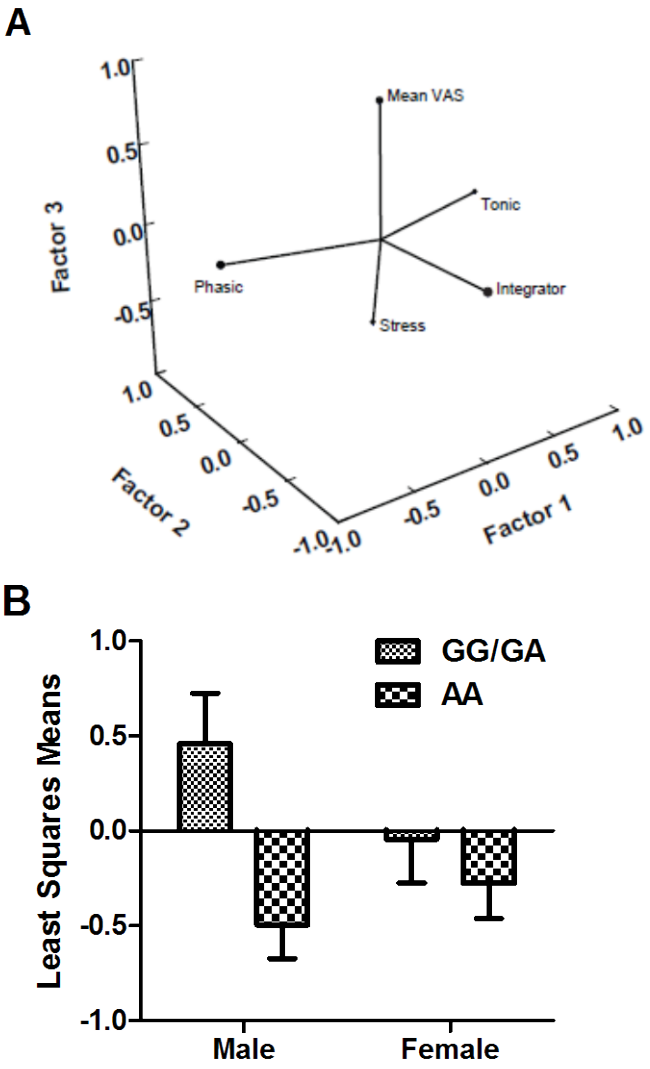

Figure S6. No effect of desmopressin (DES) on heat and pressure pain sensitivity in humans, and no interaction with stress. Absence of DES effects on heat pain thresholds (**A**) and heat pain tolerance (**B**) on the left (non-capsaicin-treated) arm for low-stress (left) and high-stress (right) groups. Symbols represent mean (± S.E.M.) pain thresholds or tolerance (º C) measured prior to drug administration and 10 and 50 min post-drug. A main effect of time was observed for both thresholds and tolerance (both $F_{1,37}$=17.5, p<0.0001), but no effects of drug nor drug x stress group interaction emerged. **C**) Absence of DES effects on pressure pain thresholds (g) on the trapezius for low-stress (left) and high-stress (right) groups at the same time points. No significant main effects or interactions were observed.

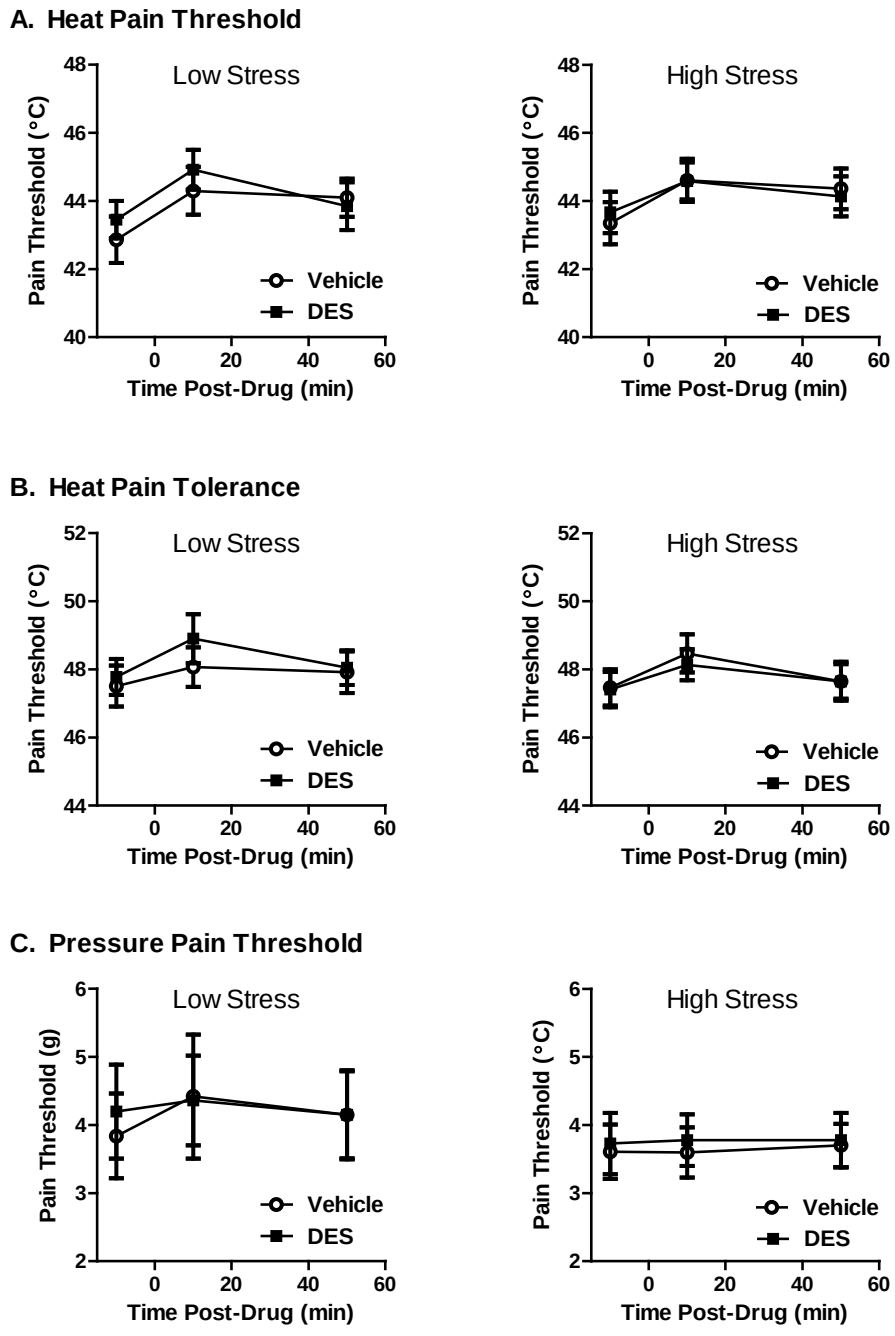

Figure S7. AVP analgesia (in habituated mice) against 2.5 g capsaicin pain is mediated via the V1AR. Bars represent mean ± S.E.M. time spent licking (s) in habituated wildtype (+/+) and *Avpr1a*$^{-/-}$ (–/–) mice (*n*=3-4/strain/drug). A two-way ANOVA revealed a significant genotype x drug interaction ($F_{1,11}$ = 11.9, p<0.001). ***p<0.001 compared to corresponding saline group.

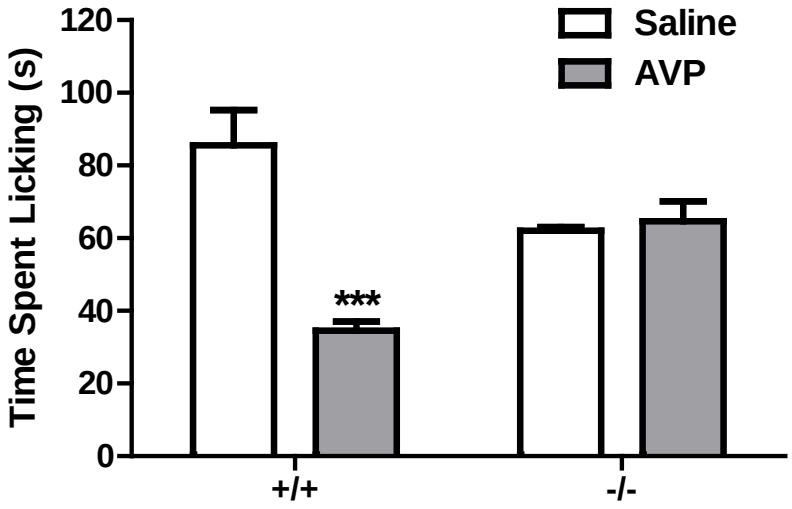

Figure S8. Pain testing in mice is stressful, and the stress is ameliorated by habituation. Reduction of stress by habituation (Hab.) compared to non-habituated (Non-Hab.) in *male* mice (**A**–**C**; *n*=4–12 mice/condition/measure) and *female* mice (**D**–**F**); *n*=6–10 mice/condition/measure) as measured by fecal boli (**A,D**), plasma concentrations of corticosterone ([CORT]; **B,E**), or plasma concentrations of AVP (**C,F)**. Bars represent mean (± SEM) total number of deposited fecal boli or mean (± SEM) trunk blood plasma CORT (ng/ml) or AVP (pg/ml) at the time of capsaicin injection; **p*<0.05. Note that emotional stressors such as novelty have been shown to *suppress* pituitary vasopressin secretion (Onaka, T. & Yagi, K., *J. Neuroendocrinol.*, 1993).

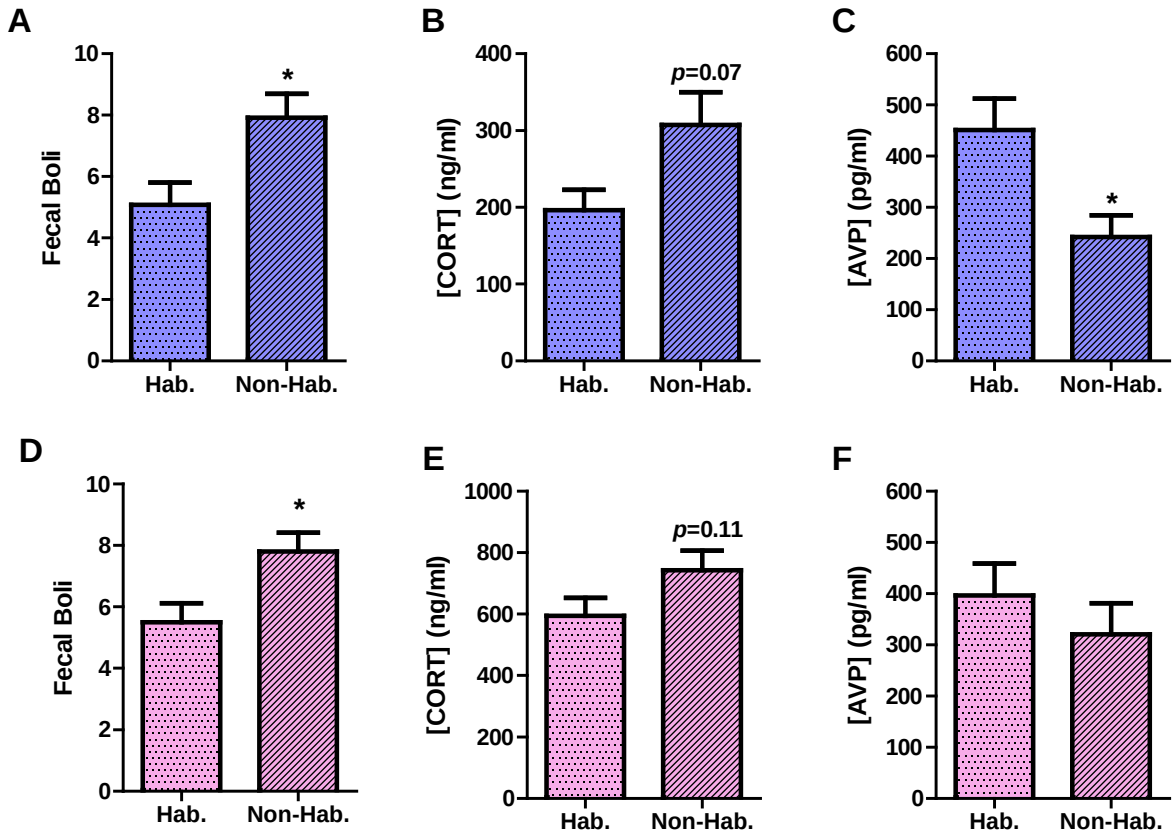

Figure S9. No interaction between AVP/V1AR-mediated analgesia and stress in female mice. Compare to Figure 5 in the main text. **A**) Female CD-1 mice (n=6–8 mice/drug/condition) either habituated or not habituated to the testing environment were tested for capsaicin (2.5 μg)-induced pain behavior following s.c. injection of AVP (0.1 mg/kg) or saline (10 ml/kg). Bars represent (± SEM) time spent licking the injected paw (s). ANOVA revealed significant main effects of habituation status ($F_{1,28}$ = 4.1, p=0.05) and drug ($F_{1,28}$ = 20.4, p<0.001), but no significant interaction ($F_{1,28}$ = 0.1, p=0.83). **B**) No differences in capsaicin sensitivity between female *Avpr1a* knockout mice (-/-) and wildtype mice (+/+) (n=3–6 mice/genotype/condition). Bars represent (± SEM) time spent licking the injected paw (s). **C**) No difference in opioid-mediated (i.e., reversible by 10 mg/kg naloxone) swim stress-induced analgesia in female *Avpr1a* knockout (-/-) compared to wildtype (+/+) mice (n=4–8 mice/genotype/drug). Bars represent mean (± SEM) percentage of maximum possible analgesia on the 56ºC hot-plate test; *p<0.05 compared to saline-treated mice of the same genotype; n.t.=not tested. **D**) A/J versus B6 strain differences in formalin sensitivity are not dependent on habituation in female mice. Bars represent mean (± SEM) percentage of positive samples in the late-phase (0-45 min) formalin test (n=6 mice/genotype/condition); *p<0.05. ANOVA revealed significant main effects of genotype ($F_{1,20}$ = 14.4, p=0.001) but not habituation status ($F_{1,20}$ = 0.2, p=0.69) or their interaction ($F_{1,20}$ = 1.0, p=0.33).

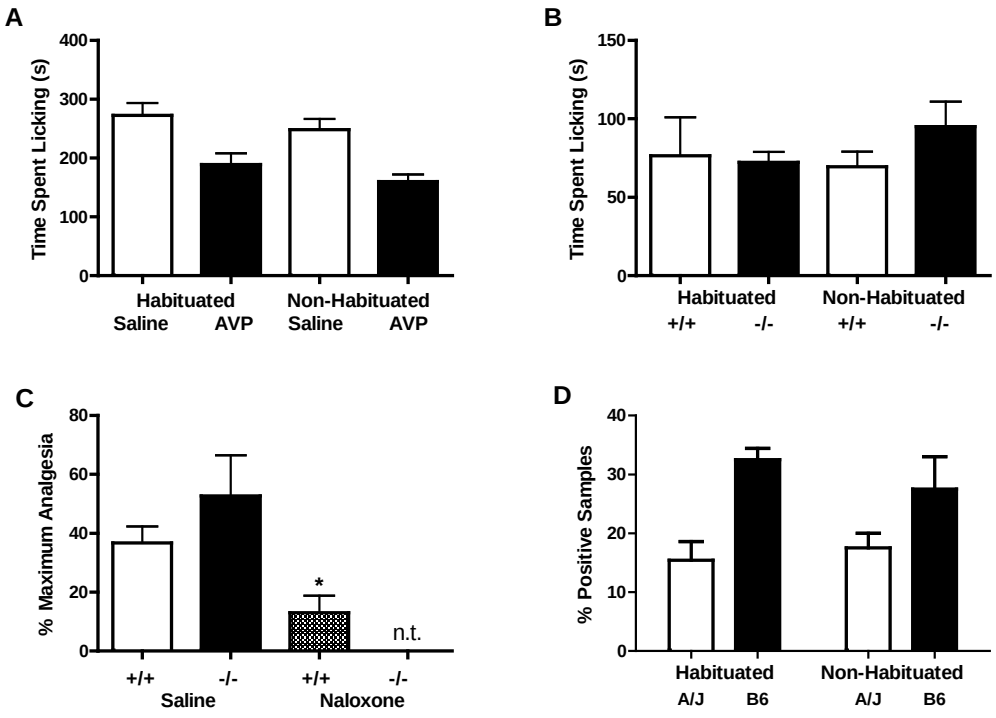

Figure S10. A model illustrating the gene-sex-environment interaction between stress, vasopressin and V1AR suggested by our data. Stress, when present (left side), activates endogenous analgesia systems involving the V1AR (middle), but the strength of V1AR activation depends on genetic factors. In genotypes with high V1AR functioning (A/J mice, *Avpr1a* +/+ mice, and rs10877969 GG/GA humans) , stress-induced analgesia is produced in males, resulting in relatively lowered pain sensitivity (bottom left). Under conditions of stress and in such individuals, whether vasopressin/desmopressin (top right) is administered or not has no impact, because the V1AR has already been activated. Only in the absence of stress (right side) can vasopressin/desmopressin analgesia (measured compared to *unaltered* basal sensitivity) be observed, and this drug analgesia (bottom right) is also mediated through the V1AR. Although the clearest relationship between stress and desmopressin analgesia was observed in rs10877969 AA males, the paucity of GG/GA subjects in the desmopressin experiment did not allow a clear evaluation of the effect of genotype on drug analgesia.

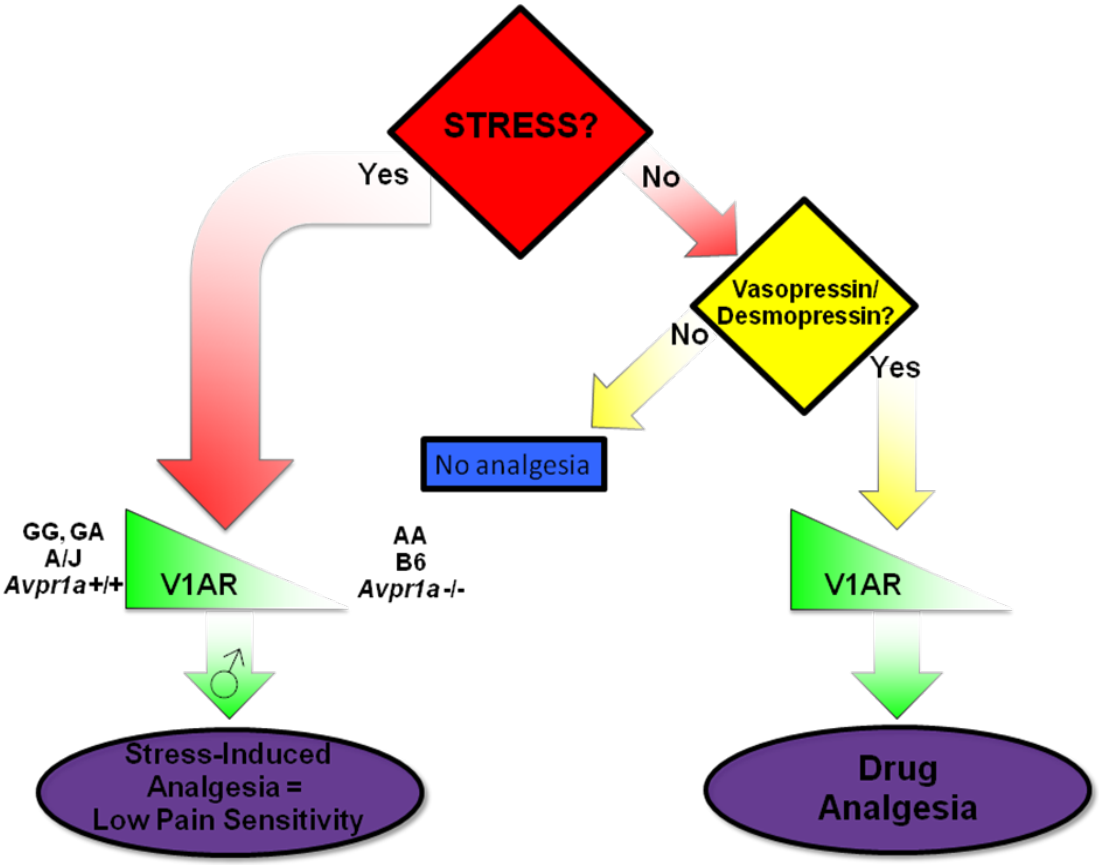

Table S1. Known transcripts on chromosome 10 between *D10Mit25* and *D10Mit103* (NCBI m37 mouse assembly).

Start Position	Gene	Protein Name	Function[a]
121,883,760	*Avpr1a*	arginine vasopressin receptor 1A	signal transduction
122,115,228	*Ppm1h*	protein phosphatase 1H (PP2C domain containing)	catalytic activity
122,420,007	D630033A02Rik	RIKEN cDNA D630033A02 gene	
122,430,527	*Mon2*	MON2 homolog (yeast)	Golgi to endosome transport
122,548,587	*Usp15*	ubiquitin specific peptidase 15	peptidase activity
122,699,173	AI851790	expressed sequence AI851790	
123,906,130	4930503E24Rik	RIKEN cDNA 4930503E24 gene	
124,290,343	LOC671416		
124,662,606	*Slc16a7*	solute carrier family 16, member 7	transporter activity
124,812,638	LOC100042987		
124,922,715	LOC100042992		

[a]Most relevant Gene Ontology Biological Process entry.

Table S2. SNPs located from 121.70–121.80 Mb on chromosome 10 showing "perfect" correspondence between late-phase formalin licking behavior and haplotype. Strains are ordered from most resistant to most sensitive, as in **Fig. 1a** of the main text.