Supporting Online Material for

Social Modulation of Pain as Evidence for Empathy in Mice

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This PDF file includes:

Materials and Methods
SOM Text
Figs. S1 to S10
References
Supporting Online Material

Materials and Methods

Subjects

Naïve, adult mice (7-13 weeks) of both sexes were used in all experiments. Studies employed CD-1® mice (ICR:Crl) purchased from a commercial supplier, shipped at 6 weeks of age, or CD-1 mice bred in-house from breeders so obtained. Shipped mice were housed together on arrival, same-sex, up to four mice per cage. Mice bred in-house were housed with same-sex littermates after weaning, and then re-caged together with same-sex non-littermates (except where noted) at 6 weeks of age. Mice were tested no less than one week after arrival or re-caging. Inbred BALB/cJ mice were used in one experiment (“Deaf” groups in Fig. 2). The vivarium was temperature-controlled and maintained under a 12:12 h light/dark cycle (lights on at 07:00 h), with ad lib access to food and tap water. Although equal numbers of mice of both sexes were used overall, when tested together only same-sex groupings were formed.

In all experiments, “Strangers” refer to mice drawn from different cages, “Cagemates” refer to mice drawn from the same cage but not necessarily related, and “Siblings” (in Fig. 1A only) refer to same-sex littermates living together from birth.
Nociceptive assays

The assays used in these experiments have been described in considerable detail previously (1). Brief descriptions are as follows, in order of appearance in the text.

**Abdominal constriction test.** In the acetic acid abdominal constriction (writhing) test, 0.9% glacial acetic acid was injected intraperitoneally (i.p.) in a volume of 10 ml/kg into mice standing on a ¼-inch-thick glass floor within Plexiglas observation cylinders (15 cm diameter; 22.5 cm high). Mice were habituated to these cylinders either alone or with the other mouse to be tested concurrently for 30-40 min before injection. Mice were briefly removed, injected, and replaced in the cylinder; in conditions where both mice received an injection, these occurred within 20 sec of each other. High-resolution video cameras located underneath the glass floor recorded activity for 30 min post-injection. Digital video files were created, archived, and later sampled for 5 sec at 20 s intervals (i.e., 90 total samples), and the presence or absence of abdominal constrictions or “writhes”—characteristic lengthwise stretches of the torso with a concomitant concave arching of the back—in that 5-s period was scored by blinded observers whenever possible. See Fig. S8 for details of this sampling method compared to conventional scoring.

**Formalin test.** In the formalin test, 1% or 5% formalin (20 µl) was injected into the plantar surface of the right hindpaw using a 50 µl Hamilton microsyringe with a 30-gauge needle. As in the writhing test, mice were standing on a glass floor within Plexiglas observation cylinders (15 cm diameter; 22.5 cm high). Mice were habituated to these cylinders with the other mouse to be tested concurrently for 30-60 min before injection. Mice were briefly removed, injected, and replaced in the cylinder;
in conditions where both mice received an injection, these occurred within 40 sec of each other. Video cameras located underneath the glass floor recorded activity for 60 min post-injection. Digital video files were sampled for 5 sec at 1-min intervals (i.e., 60 total samples), and the presence or absence of recuperative behavior—licking or biting of the affected hindpaw—in that 5-s period was scored by blinded observers whenever possible. Sampling of formalin test behavior in this way has been shown by ourselves and others to be accurate and reliable (2, 3).

Paw-withdrawal test. In the radiant heat paw-withdrawal test (4), mice were placed on a glass floor within Plexiglas observation cylinders (15 cm diameter; 22.5 cm high). Mice were habituated with the other mouse to be tested concurrently for at least 2 h before testing commenced; the longer habituation time was necessary to reduce activity levels such that the paw-withdrawal test could be performed. The stimulus was a high-intensity beam (Setting = 3, ≈45 W) from a projector lamp bulb located 6 cm below the glass floor, and was aimed at the plantar surface of the mid-hindpaw of an inactive mouse. Paw-withdrawal latency was measured, to the nearest 0.1 s, on both hindpaws (separated by 20 - 30 s) before, and every 5 min after injection of 0.9% acetic acid for 30 min. Two determinations per hindpaw were made for baseline measurements, and one determination per hindpaw for post-injection measurements. No laterality effects were noted, so data from both hindpaws were averaged at every time point.

Sensory disruptions

Smell. Anosmia was produced in mice by intranasal application of zinc sulfate (ZnSO₄), which destroys the olfactory epithelium (5). A recent study in the mouse using...
olfactometric testing confirmed the dramatic and virtually complete anatomical
deafferentation of the main olfactory bulb and consequent profound anosmia from 4 - 8
days after treatment (6). CD-1 mice were treated with 2 - 3 drops of the local anesthetic
lidocaine, and 5 min later 50 µl of 5% ZnSO₄ (or saline) was injected into each nostril
using a blunted, 4-mm-long, 26-gauge needle. Anosmia was confirmed behaviorally at
3 - 4 days post-treatment by assessing latency of food-deprived mice to detect a piece of
mouse chow buried 0.5 cm under the bedding of a novel cage. Saline-treated mice
retrieved the food in 30.9 ± 4.5 s; mice were considered anosmic if their latency exceeded
120 s (two ZnSO₄-treated mice were discarded based on this criterion). Twenty-four to
48 h later, all mice were tested on the writhing test as described.

**Hearing.** We rendered mice deaf using a chemotoxic strategy. Systemic injection of
the aminoglycoside kanamycin produces ototoxicity and profound shifts in auditory
thresholds across the frequency spectrum. This irreversible effect is strain-dependent in
the mouse, with the largest changes (up to 70 dB at 24 kHz) seen in BALB/c mice (7).
The pathology follows the expected base-to-apex pattern, with greater threshold shifts
at higher frequencies (7), and so audition in the ultrasonic range would be greatly
impaired. BALB/cJ mice were given two weeks of twice-daily injections of 800 mg/kg
(s.c.; 10 ml/kg volume) kanamycin base, and tested one week later on the writhing test
as described. Mice were tested for startle response to loud, high-frequency sounds,
and found to be greatly impaired compared to untreated mice.

**Touch.** We prevented tactile contact between dyadic mice by placing a 20-cm-high,
1/8-inch-thick, transparent Plexiglas divider raised 2 mm off the floor between them
(“Transparent” condition in Fig. 2A). This barrier allowed visual and pheromonal
Vision. We prevented visual contact between dyadic mice by placing a 20-cm-high, 1/8-inch-thick, opaque (grey) Plexiglas divider raised 2 mm off the floor between them ("Opaque" condition in Fig. 2A). This barrier allowed only pheromonal contact, also preventing tactile contact.

Statistical analyses

Statistical tests are described in the main text and SOM figure legends. In every case a criterion $\alpha = 0.05$ was adopted.
SOM Text

_Socially mediated pain hypersensitivity in non-genetically related mice_

Mice were ordered from a commercial supplier (Charles River Canada, Boucherville, QC) with instructions to send four siblings each from six separate litters showing the minimum possible genetic relationship (i.e., second cousins or further). Mice were identified by ear punch before delivery. Upon arrival, mice were re-caged to separate siblings from each other, such that each mouse in a cage was as genetically unrelated as possible to each other mouse. Mice were co-housed for 28 days before testing. As can be seen in Fig. S1, the BW vs. OW hyperalgesia persisted.

_Observation of videotaped behaviors in mouse dyads in the writhing test_

All mice whose data form Fig. 1 in the main text were digitally videotaped at the time of testing, and those files were archived. A pseudorandom sample of those files (featuring both OW and BW dyads) was later analyzed carefully by blinded observers for quantifiable behaviors other than writhing. In addition, separate groups of 21-day cagemate and stranger mice were placed together and videotaped in “no writhing” (NW) dyads for examination of contact behaviors in the absence of pain.

From a behavioral perspective, the subjects, in addition to being tested for pain sensitivity, were simultaneously participating in a modified open field test (8) and in a modified social interaction test (9). The present situation differs from the classic implementation of these tests in a number of ways, however. Open fields used for measurement of anxiety (or stress, or emotionality) (10) are typically much larger than
the observation cylinders used here (≈1 m in diameter vs. 15 cm presently). This makes scoring thigmotaxis (wall hugging) and center-square entries impossible, and greatly reduces the incidence of both rearing and freezing, leaving only defecation as a measure of anxiety (10). In the classic social interaction test, rodents are placed in a familiar or unfamiliar arena, and the amount of active social interaction between them (olfactory investigation, allogrooming, following, crawling over/under, and aggressive behaviors) provides an index of their anxiety level. Typically, in tests of sociability, the scored behaviors are found most often in the first 5-10 minutes of social exposure (9, 11).

Our videotapes, by contrast, show behavior beginning 30 min after the initial placement of the two mice in the observation cylinder. As a result, most active social interactions had likely already occurred; the remaining social interactions were mostly passive (i.e., body contact). It should be noted that the presence of acetic acid injections would confound any standard implementation of existing social interaction tests, as would the very small size of the observation cylinders, since mice cannot avoid their neighbor. Nonetheless, a number of important behaviors could be quantified, as described below.

**Locomotor activity.** Locomotor activity within the observation cylinders was estimated by dividing the cylinder into quadrants (via imaginary lines on the video screen), and counting the number of quadrant entries (defined by half-body “line” crossings) by each mouse in the 30-min testing period. As shown in Fig. S2, locomotor activity was equivalent across all conditions (one-way ANOVA: $F_{6,65} = 1.0, p = 0.44$).

**Fighting.** In 83 videos so scored (44 female dyads, 39 male dyads; 41 cagemate/sibling dyads, 42 stranger dyads), only two instances of fighting were observed: in one male-cagemate-OW dyad and one male-stranger-OW dyad. The rarity of fighting or any
other aggressive behavior is likely due to the fact that both mice were exposed to the “neutral” novel chamber simultaneously; that is, the situation does not resemble the resident-intruder paradigm used to study aggression (12).

**Physical contact.** Physical contact was scored by subjective judgment of a blinded observer; mice were said to be in physical contact if any parts of their bodies (excluding whiskers or tail) (13) were touching at any point during the 5-sec sample. Mice in dyads, whether pain was present or not, spent approximately half of their time in physical contact with each other, including huddling (see Fig. S3). A two-way ANOVA (familiarity x dyadic condition) revealed only a main effect of condition ($F_{2,133} = 4.5$, $p < 0.05$), with a Tukey post-hoc test revealing that mice in the OW condition spent significantly more time in physical contact than mice in the BW condition. We speculate that mice in the BW condition might be preoccupied with their own pain, and thus less willing to engage in social behavior. As shown in Fig. S3, the only suggestive difference between cagemate and stranger dyads is in the OW condition, with cagemates showing some evidence for increased contact time. This difference was actually significant by $t$-test ($p < 0.05$), but the familiarity x condition interaction failed to reach significance ($F_{2,133} = 1.5$, $p = 0.20$). There was no significant correlation between percentage contact and writhing totals in any group ($r$’s = -0.38 – 0.17).

We also investigated which of the two mice *initiated* each contact in a subset of OW videos. For both cagemates and strangers, contact was initiated equally by the writher (49.3 ± 4.6% and 46.6 ± 4.5%, respectively) and the non-writher (50.7 ± 4.6% and 53.4 ± 4.5%, respectively).
**Defecation.** The number of fecal boli deposited by the two mice in the dyad were counted. In this particular case we were able to distinguish defecation during the 30-min habituation phase (i.e., due to novelty and social stress) from defecation during the 30-min writhing test (i.e., due to pain), simply by counting boli at the beginning of the video and again at the end. It was not possible to distinguish the number of fecal boli deposited by individuals in each dyad, however, so total boli counts were divided by two. As shown in Fig. S4A, there was no significant difference between groups in boli counts at the end of the habituation period (one-way ANOVA: $F_{4,98} = 1.7, p = 0.16$), with all groups having deposited 6-8 boli on average. In isolated mice and cagemate dyads, no additional boli were deposited on average during the 30-min period after acetic acid injection. Mice in stranger dyads, by contrast, deposited additional boli in this period (Fig. S4B).

Defecation is a common and well-validated measure of anxiety in the open field test (10). These data therefore suggest that mice tested for pain in the presence of strangers experience more stress/anxiety than mice tested for pain alone or in the presence of cagemates. These findings suggest that the known phenomenon of stress-induced hyperalgesia (14-16) is unlikely to account for the socially mediated pain hyperalgesia described in the main text, since that phenomenon was seen in cagemates but not strangers.

**Sex-specificity of writhing inhibition among stranger dyads**

We conducted analyses of the data shown in Fig. 1 separated by sex; these data are illustrated in Fig. S5A. As can be seen, sex had no overall influence on writhing
behavior in isolated mice or on the extent of hypersensitivity in the BW condition.

The lack of a sex difference in hypersensitivity is perhaps surprising given that in
humans, women consistently score higher on measures of empathy than men (17, 18).

The apparent species difference may lend credence to the hypothesis that sex differences
in empathy are socioculturally based. In only one case did sex interact with condition:
we observed a significant decrease in writhing behavior in males but not female strangers
tested in the OW condition. Thus, data from male mice accounted for the entire decrease
in this condition shown in Fig. 1. A further analysis of the male data revealed an
apparent bimodality, in which half of the male-Stranger-OW mice displayed virtually no
writhing at all (Fig. S5B).

Synchronization and variance reduction of licking behavior in the 5% formalin test of
mice tested simultaneously

This study involved the testing of 203 B6AF2 mice for the purposes of quantitative
trait loci identification, and the data have been published previously (19). All mice were
injected in the right hindpaw with 5% formalin (20 µl, intraplantar) and videotaped
conventionally for 90 min post-injection. Almost all mice (n = 180) were tested in
groups of four (2 males; 2 females) per experimental run, with each mouse placed singly
within transparent Plexiglas cylinders located approximately 20-25 cm apart. Mice tested
within a run were almost always siblings, and half of them (i.e., same-sex siblings)
were cagemates since weaning as well (i.e., for at least 4 weeks). Data from this
experiment were recorded in time bins of 5 min each over a 90-min period, and thus
synchrony among mice tested together could be estimated by the correlation of behavior
Langford et al. Social modulation of pain as evidence for empathy in mice

(i.e., licking duration) across 5-min time bins. We calculated all six correlations between four mice in a particular run, then took the average of those correlations ($R$; see Fig. S6 for an explanatory graphic), and then calculated the grand average of $R$ values across the 45 experimental runs. This grand average correlation, shown in Fig. S7A (in blue), was 0.27 ± 0.02. Of course, due to the known time-dependence of the formalin test (i.e., the “early phase,” “interphase” and “late phase” showing high, low and high licking behavior, respectively) (20), one would expect a non-zero correlation. We thus took the data and permuted it 100 times, forming random groupings of four mice per “run,” and calculated correlations as described above. The frequency histogram of the 100 permutations is shown in Fig. S7A. The actual grand average correlation among mice tested together (in real life) had a significant $z$-score of 3.2 compared to the permutations, demonstrating that mice tested together were statistically synchronized in their licking behavior. Moreover, we found in this data set that not only was licking behavior synchronized in time, but overall levels of behavior converged (i.e., the between-subject variance was reduced) such that the actual standard deviation of overall licking totals of the four mice within each run was significantly lower ($z = -2.9$; again, compared to permuted data) than that of randomly grouped mice (see Fig. S7B).

Although these findings are strongly suggestive of the possibility that mice within an experimental run were affecting each other’s behavior—even though they were tested singly in separate cylinders, albeit in visual contact with one another—it is possible that the variance reduction was due to mice within a run sharing environmental determinants affecting formalin test sensitivity. To disambiguate these two possibilities, we performed the experiment shown in Fig. 3 of the main text. That study clearly shows that the
behavior of one mouse can directly affect, in either direction, the behavior of a neighboring (and familiar) mouse.

**Increased reliability of sampling-based scoring on the abdominal constriction test**

The conventional method of scoring the writhing test is to count the number of “writhes” in a 15-30 min period following the intraperitoneal injection of a chemical irritant. Normally this is done “live,” but our recent switch to digital video recording has allowed the assessment of inter-rater reliability. We find (Fig. S8A) that inter-rater reliability is quite poor, likely due to the difficulty of deciding whether writhes with fluctuating “obviousness” actually represent a single long writhe or multiple shorter writhes. Sampling offers a solution to this problem, because one need not ask when a writhe begins or ends but rather simply whether a mouse is writhing at any particular moment in time. As shown in Fig. S8B, a sampling strategy where 5 s of every 20-s time period is assessed for the presence or absence of writhing yields data that are accurate compared to scoring writhing counts ($r = 0.69-0.90$), and have far higher inter-rater reliability ($r = 0.94$ vs. $r = 0.64$ for sampling and counting, respectively).

**No bidirectional modulation of formalin licking behavior in strangers**

Fig. S9 shows the absence of significant modulation of licking behavior in strangers ($n = 20$/condition). Methods were exactly as described for the cagemate data shown in Fig. 3.

All groups displayed the expected biphasic pattern of responding. A two-way (injected dose x observed dose) repeated measures ANOVA revealed a significant main
effect of injected dose and a significant injected dose x repeated measures interaction (both $p$’s < 0.001), but not main effects or interactions involving observed dose.

Considering the total licking data from 0-40 min post-injection, ANOVA revealed a highly significant main effect of injected dose ($F_{1,76} = 17.9, p < 0.001$) and a marginal effect of observed dose ($F_{1,76} = 3.2, p = 0.08$); the injected dose x observed dose interaction was not significant.

No cross-modality hyperalgesia in strangers

Fig. S10 shows that the observation of writhing in a stranger mouse does not significantly alter thermal pain sensitivity ($n = 16 – 18$/condition). Methods were exactly as described for the cagemate data shown in Fig. 4. As can be seen, only the OW-Inj. group displayed significant hyperalgesia compared to baseline; there was no evidence of hyperalgesia produced by observation alone (in either OW-Uninj. or BW groups).

It should be noted that the thermal hyperalgesia induced in strangers (Fig. S10) and cagemates (Fig. 4) by acetic acid injection is actually in contrast to expectations from a large literature describing the phenomenon of diffuse noxious inhibitory controls (DNIC) (21, 22), whereby a noxious stimulus delivered to one part of the body produces analgesia in another location. DNIC is proposed as the mechanism underlying the long-known phenomenon of counter-irritation analgesia. We have no convincing explanation for why we observe hyperalgesia and not analgesia in these experiments. We note that our observations are robust and have been replicated several times, and we do not believe that anyone has ever demonstrated DNIC with the use of acetic acid as the conditioning stimulus and thermal latencies as the test stimulus.
Figure Legends

Fig. S1. Socially mediated hyperalgesia in the absence of close genetic relatedness (see SOM text above for details). Mice \((n = 11-12/\text{condition})\) were tested as described in Fig. 1. Bars represent mean ± S.E.M. percentage of sampled intervals showing writhing behavior (% Samples Writhing). *\(p < 0.05\) compared to OW group (\(t\)-test).

Fig. S2. Locomotor activity within observation cylinders in the 30-min period following injection of 0.9% acetic acid. Mice \((n=10-12/\text{condition})\) were tested alone (Isolated), or in dyads with strangers or cagemates. Either one mouse in the dyad was given acetic acid (OW-Inj.) and one was not (OW-Uninj.), or both mice were given acetic acid (BW). Bars represent mean ± S.E.M. number of quadrant entries. See SOM text above for statistical analysis details.

Fig. S3. Physical contact between mice tested in dyads with or without acetic acid (group sample sizes are indicated in italics). In the NW group, neither mouse received acetic acid; in the OW group, one mouse only received acetic acid; in the BW group, both mice received acetic acid. Bars represent mean ± S.E.M. percentage of samples (5 sec of every 20 sec) in which mice were judged by a blinded observer to be touching each other. *\(p < 0.05\). See SOM text above for statistical analysis details.

Fig. S4. Defecation associated with social interaction and acetic acid (group sample sizes are indicated in italics). Bars in A represent mean ± S.E.M. total number of fecal boli on
the floor of the observation cylinder 30 min after mice were placed in observation chambers, and immediately prior to acetic acid injection. Bars in B represent mean ± S.E.M. additional boli deposited in the 30 min following acetic acid injection. *p<0.05 compared to zero (one-sample t-test) and to Isolated and Cagemate groups (ANOVA).

Fig. S5. A reanalysis by sex of the data shown in Fig. 1. In graph A, mice of both sexes (M: male; F: female) were tested in isolation (Isolated), or in dyads where either one mouse (One Writhing; OW) or both mice (Both Writhing; BW) received acetic acid injections. Bars represent mean ± S.E.M. percentage of sampled intervals showing writhing behavior (% Samples Writhing). Group sample sizes are indicated in italics. *p < 0.05, **p < 0.01; ***p < 0.005 by Dunnett two-way case-control comparison posthoc test compared to Isolated mice. Graph B is a frequency histogram of data from male-Stranger-OW mice only.

Fig. S6. The 5% formalin test shows a biphasic time course overall (left graph; blue symbols represent mean licking time per 5-min post-injection of formalin), but individual mice display bouts of licking behavior (left graph; individual data from four mice tested together in the same run). To estimate whether these bouts were random or synchronized, we calculated the six possible correlations between the time-courses of four mice tested together in a particular run ($r_1$ to $r_6$ in right graph), and estimated the within-run synchronization as the average of those correlations ($R$).

Fig. S7. Graph A is a frequency histogram of within-run $R$ (see Fig. S6) estimating the degree of time-synchrony of licking across 5-min time bins in “runs” of four mice formed
by 100 random permutations of the real data. The actual mean $R$ of mice actually tested together in experimental runs is shown in blue, and is significant ($z = 3.2$). Graph B is a frequency histogram of the standard deviations (SDs) of the total licking behavior of four mice in “runs” formed by 100 random permutations of the real data. The mean SD of mice actually tested together is shown in blue, and is significant ($z = -2.9$).

Fig. S8. Writhing data from Fig. 1 ($n = 43$; a randomly chosen subset) scored either conventionally (in A) or by sampling (in B) by two experimenters, SEC and DL. Scoring by sampling was accurate compared to counting writhes, and with far superior inter-rater reliability (compare $r$-values in graphs A,B).

Fig. S9. No bidirectional modulation of pain behavior produced by observation of a stranger in the formalin test. Mice, all strangers, were tested in dyads as described in Fig. 3. In the “Same” condition both mice received either 1% formalin or 5% formalin. In the “Different” condition one mouse received 1% formalin and the other received 5% formalin. Graph A shows data from all mice receiving 1% formalin; the legend describes the status of the other mouse in the dyad. Graph B shows data from all mice receiving 5% formalin; the legend describes the status of the other mouse in the dyad. In graphs A and B (note different ordinate scales), symbols represent mean ± S.E.M. percentage of sampled intervals showing formalin-induced recuperative behavior (% Samples Licking) per 5-min time bin. Graph C shows totals in all conditions from 0-40 min post-injection. *$p < 0.05$ compared to analogous 1% condition. See SOM text above for statistical analysis details. Compare to Fig. 3 of the main text, in which cagemates were tested.
Fig. S10. No thermal hyperalgesia produced by observation of a stranger injected with acetic acid. Mice (all strangers) were tested in dyads as described in Fig. 1. Prior to injection, all mice were tested for baseline thermal sensitivity. In the BW (“both writhing”) group, both mice were removed at time = 0, given an injection of 0.9% acetic acid, and returned to their cylinder. In the NW (“none writhing”) group, both mice were removed and replaced with neither receiving any injection. In the OW (“one writhing”) group, one mouse received an acetic acid injection (OW-Inj.) and the other (OW-Uninj.) did not. All mice were retested for thermal sensitivity at 5-min intervals for 30 min. Symbols in graph A represent mean ± S.E.M. paw-withdrawal latencies (average of both hindpaws). Bars in graph B represent mean ± S.E.M. average change in paw-withdrawal latencies from the baseline latency. **p<0.01 compared to zero (one-sample t-test). ANOVA revealed no significant differences across condition. Compare to Fig. 4 of the main text, in which cagemates were tested.
Figure S1
Figure S2
Figure S3
Figure S4

A. After Habituation

B. After Acetic Acid
Figure S5

A

![Graph showing % Samples Writhing](image)

- **Isolated**
- **OW**
- **BW**

Sex: M  F   M  F   M  F
Relation: Strangers  Cagemates  Sibs

B

![Graph showing Count vs % Samples Writhing](image)
Figure S6
Figure S7
Figure S8

A) Writhing Counts

B) % Samples Writhing

$r = 0.64$

$r = 0.94$
Figure S9

A. 1% Formalin

B. 5% Formalin

C
Figure S10

(A) Paw-withdrawal latency (s) over time post-injection (min) for NW, OW-Inj., OW-Uninj., and BW conditions. Error bars represent standard error of the mean.

(B) Change in paw-withdrawal latency (Δ) for NW (Uninj.), OW (Inj.), and BW conditions. ** indicates statistical significance.
References
