BEHAVIOR ECOLOGY

Adding up the odds—Nitric oxide signaling underlies the decision to flee and post-conflict depression of aggression

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Fighting is dangerous, which is why animals choose to flee once the costs outweigh the benefits, but the mechanisms underlying this decision-making process are unknown. By manipulating aggressive signaling and applying nitrergic drugs, we show that the evolutionarily conserved neuromodulator nitric oxide (NO), which has a suppressing effect on aggression in mammals, can play a decisive role. We found that crickets, which exhibit spectacular fighting behavior, flee once the sum of their opponent's aversive actions accrued during fighting exceeds a critical amount. This effect of aversive experience is mediated by the NO signaling pathway. Rather than suppressing aggressive motivation, NO increases susceptibility to aversive stimuli and with it the likelihood to flee. NO's effect is manifested in losers by prolonged avoidance behavior, characteristic for social defeat in numerous species. Intriguingly, fighting experience also induces, via NO, a brief susceptible period to aversive stimuli in winners just after victory. Our findings thus reveal a key role for NO in the mechanism underlying the decision to flee and post-conflict depression in aggressive behavior.

INTRODUCTION

Competition between conspecifics has driven animals to evolve fighting strategies that are optimized to secure limited resources at minimal cost (1). But how do animals know when better to flee rather than persist? Despite numerous behavioral theories (2, 3), and insights into the neurochemical control of aggression in both vertebrates (4, 5) and invertebrates (6, 7), the mechanisms underlying the decision-making processes are barely known. Most behavioral assessment theories agree that the decision is based on information gathered from agonistic signals exchanged during fighting (2, 8), but it is hotly debated who evaluates these signals (sender, receiver, or both), how they act on aggression (promote or suppress), and whether complex cognitive capacities are required (3, 9–11). Our earlier experiments (12) revealed that crickets simply add up the sensory impact of their opponent's agonistic signals during fighting, and flee when the sum exceeds some critical threshold, which is in full accord with the cumulative assessment model (8). Here, we investigate the role of the nitric oxide/cyclic guanosine 3′,5′-monophosphate (NO/cGMP) signaling pathway in opponent assessment. Although NO acts to suppress aggression in mammals and is implicated in inappropriate aggression in human mental disorders (4, 13, 14), its specific behavioral function in normal aggressive behavior is unknown (13). The insect NO/cGMP signaling pathway is similar to that in mammals (15), but it is unclear whether this promotes (16) or suppresses aggression (17). Our study reveals that the sensory impact of aversive experiences during fighting activates the NO signaling pathway, which promotes the decision to flee and leads to post-conflict depression of aggression that is characteristic for social defeat in many animal species (18).

RESULTS

Cricket fights are spectacular affairs, characterized by a stereotyped sequence of increasingly aggressive acts or levels of aggression (fig. S1)

(19, 20). Under control conditions, equally sized males deprived of social contact for 24 hours ("naïve") that received no drugs (21) or only control solutions (Fig. 1) typically fight for 7 s until one, the loser, retreats and thereby establishes the winner. Fighting is initiated by antennal contact and starts with an antennal fencing match (level 2), followed by mandible threat displays of one then both opponents (levels 3 and 4, respectively), and typically culminates in mandible engagement (level 5) but rarely escalates to grappling matches (level 6). In comparison, fights were significantly less fierce when both opponents were treated with activators of the NO/cGMP signaling pathway including the NO donor S-nitroso-N-acetyl-DL-penicillamine (SNAP: 5 mM, $n = 80$, U tests versus Ringer-a control: P_{level} and $P_{duration} < 0.001$) and 8-bromo cGMP (8Br-cGMP), a membrane-permeable analog of cGMP (1 mM, $n = 95$, U tests versus Ringer-b: $P_{level} = 0.038$, $P_{duration} = 0.04$; Fig. 1, red bars). Conversely, fights escalated to significantly higher levels, often involving grappling (level 6), and lasted far longer when the crickets received inhibitors of the NO/cGMP pathway (Fig. 1, blue bars). The inhibitors included the NO scavenger 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO; 10 mM, $n = 91$, U tests versus Ringer-c: $P_{level} = 0.0042$, $P_{duration} = 0.0003$), the competitive NOS inhibitor N^{ω}-nitro-L-arginine methyl ester hydrochloride (LNAME; 20 mM, $n = 111$, U tests versus its non-effective enantiomer DNAME: P_{level} and $P_{duration} < 0.0001$), or the irreversible inhibitor of soluble guanylyl cyclase 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one [ODQ; 1 mM in 1% dimethyl sulfoxide (DMSO) in Ringer, U tests versus DMSO: $P_{level} = 0.0125$, $P_{duration} = 0.0179$]. The aggression-promoting effects of the inhibitors were also additive so that crickets that received both PTIO and LNAME often fought exceptionally long (25% > 40 s, $n = 40$, dosages as previous).

Treatment with nitrergic drugs before a fight (Fig. 1) also had a long-lasting influence on subsequent interactions (Fig. 2). As in many species (18), crickets that lose a contest remain submissive and retreat from any approaching male (level 1) for some 3 hours before regaining their initial aggressive state (22). Confirming earlier suggestions (17), our data show that post-conflict depression of aggression results from activation of the NO/cGMP pathway (Fig. 2A and fig. S2). In our experiments, the losers of fights between drug-treated crickets (cf. Fig. 1) were rematched against their aggressive victors at different times after defeat (Fig. 2). As

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opposed to controls, SNAP- or 8Br-cGMP–treated losers failed to fully regain their aggressiveness within 3 hours (U tests versus Ringer-a, SNAP: $P_{level} = 0.014$, $P_{duration} = 0.026$, versus Ringer-b, 8Br-cGMP: $P_{level} = 0.037$, $P_{duration} = 0.0014$; Fig. 2A), but recovered completely 12 hours later (Wilcoxon signed-rank test, SNAP first fight versus SNAP 12 hours after defeat: $P_{level} = 0.429$, $P_{duration} = 0.223$, data not illustrated). Contrasting
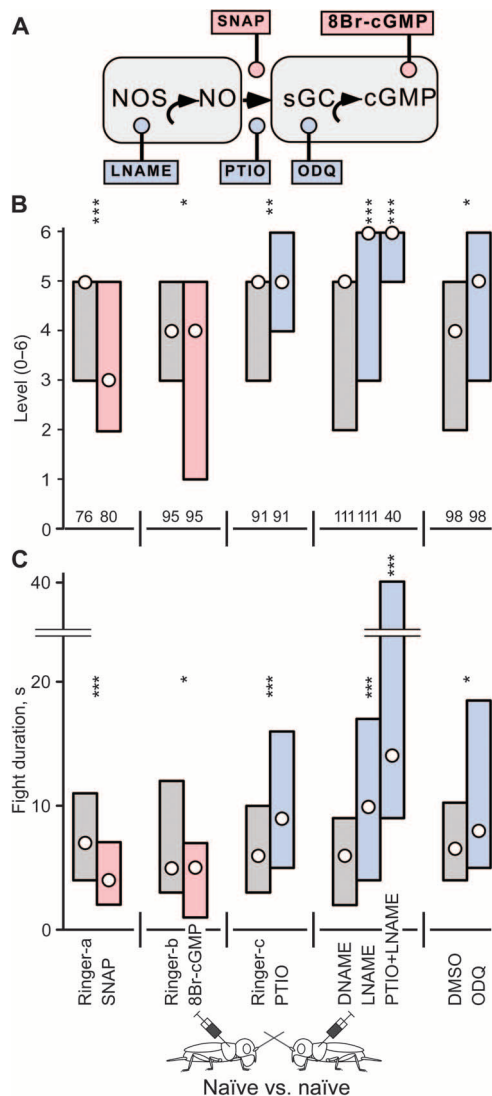
Fig. 1. The NO/cGMP signaling pathway reduces the expression of aggression in socially naïve crickets. (**A**) Pictogram illustrating the site of actions of the drugs. Activators (red): SNAP, an NO donor; 8Br-cGMP, a tissue-permeable analog of cGMP. Inhibitors (blue): LNAME, an NO synthase (NOS) inhibitor; PTIO, an NO scavenger; ODQ, an irreversible inhibitor of soluble guanylyl cyclase (sGC). (**B** and **C**) Bar charts comparing the level of aggression and fight duration, respectively, for size-matched, socially naïve contestants that were both treated with NO/cGMP activators (red bars: SNAP and 8Br-cGMP), inhibitors (blue bars: PTIO, LNAME, LNAME + PTIO, and ODQ), and separate appropriate controls (gray bars: Ringer-a, b, c, DNAME, and DMSO, respectively). Circles, median; bars, interquartile range (IQR); n on x axis in (B) gives the number of contests. Significant differences to control groups are indicated by asterisks (U test, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; exact P values given in text).
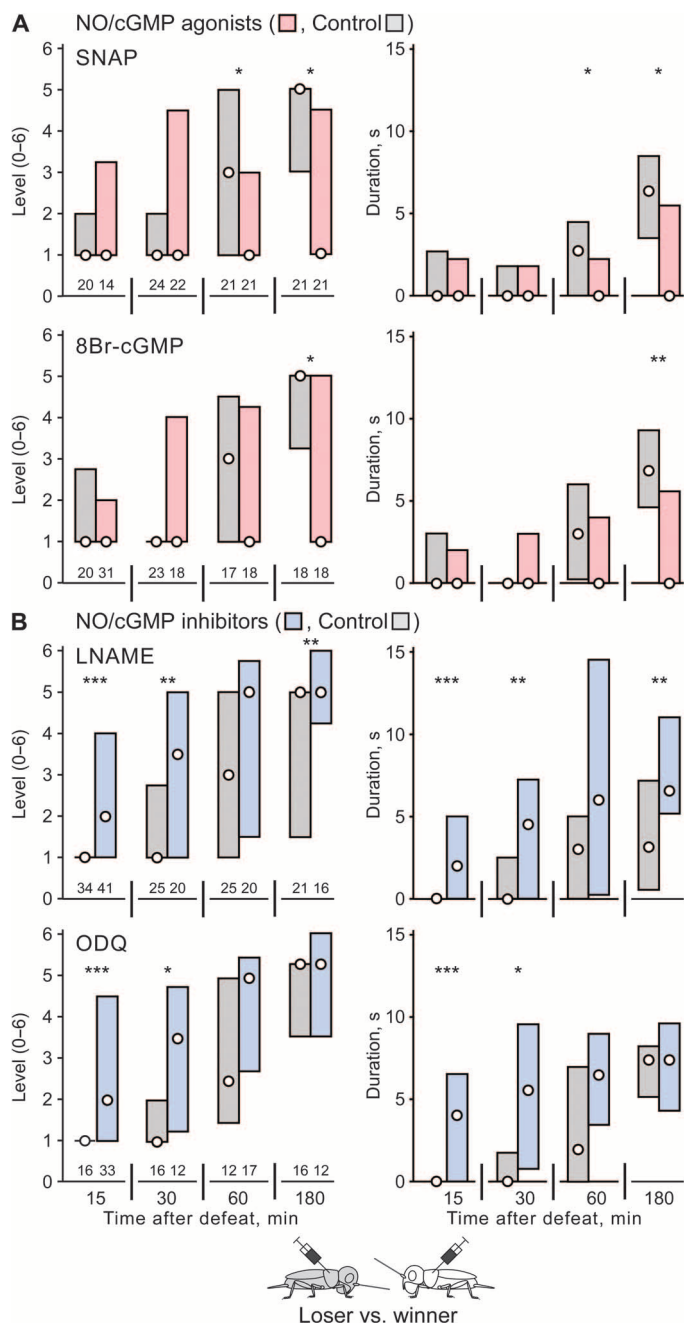

Fig. 2. The NO/cGMP pathway mediates depressed expression of aggression after social defeat. Bar charts comparing the level (left) and duration (right) of aggressive interactions between losers matched against their previous victors at different times after defeat (different animals for each time slot; circles, median; bars, IQR; n is given on left x axes). (**A** and **B**) Each pair of contestants was drug-treated before the initial (first) contest (cf. Fig. 1): (A) NO/cGMP activators (red bars: SNAP and 8Br-cGMP) and (B) inhibitors (blue bars: LNAME and ODQ). The effect of each drug is compared to its appropriate control (gray bars: Ringer-a for SNAP, Ringer-b for 8Br-cGMP, DNAME for LNAME, and DMSO for ODQ). Significant differences are indicated by asterisks (U test, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Stevenson *et al. Sci. Adv.* 2015;1:e1500060   13 March 2015

2 of 7

this, losers treated with NO/cGMP inhibitors regained their aggressiveness within only 15 min (U tests: LNAME versus DNAME and ODQ versus DMSO: P_{level} and $P_{duration}$ < 0.001 for both; Fig. 2B). As summarized in fig. S2, the time course of loser recovery was similar in all control groups and essentially complete within 3 hours (Ringer, DNAME, or DMSO), by which time SNAP- and 8Br-cGMP–treated crickets recovered to only 50%, whereas LNAME-, PTIO-, and ODQ-treated crickets recovered by 60% within 15 min.

This led us to ask whether the NO-signaling pathway directly suppresses the tendency to fight (that is, aggressive motivation) or alternatively promotes the decision to flee by mediating the effect of the opponent's agonistic signals [cf. (12)]. To tease these two possibilities apart, only one contestant of each competing pair was drug-treated, and one or both were handicapped to impede the transmission and/or perception of agonistic signals. We first verified that crickets with either blackened eyes ("blind") or immobilized mandibles ("disarmed") engaged nonhandicapped crickets with unabated fight intensity, duration, and win chances, irrespective of whether treated with no drug [cf. (12)] or drug-control solutions (Ringer, Fig. 3A; DNAME, Fig. 3B, gray bars). In these control experiments, the blind contestants also engaged disarmed opponents without any decrement in escalation level or fight duration; remarkably, however, the blind crickets practically always won (87%; $n = 23$; χ^2 compared to 50%, 7.3; $P = 0.007$; Fig. 3). This illustrates that crickets conform to the cumulative assessment model (8) because only the opponent's actions influence the decision to flee [see (12) for supporting experiments and arguments]. Accordingly, blind always beat disarmed, because they receive no visual and reduced physical signals from opponents lacking functional mandibles, whereas disarmed accumulate the full brunt of blind's visual and physical actions and hence become the first to flee. This now puts us in a position to evaluate the influence of nitrergic drugs on the process of opponent assessment.

Compared to vehicle, SNAP-treated crickets matched against untreated opponents escalated less, fought shorter, and won less often (U tests versus Ringer: $P_{level} = 0.0006$, $P_{duration} = 0.0005$; win chances: SNAP 21%, $n = 24$; Ringer 49%, $n = 33$; $\chi^2 = 4.567$, $P = 0.032$; Fig. 3A). Significantly, however, when deprived of visual inputs, blind SNAP-treated crickets fought as harsh and as long as controls (Ringer), irrespective of whether against untreated or disarmed opponents. Hence, NO is not necessarily reducing the tendency to fight per se. Supporting this, crickets treated with the NOS inhibitor LNAME did not escalate significantly more or fight longer than controls (DNAME) against untreated or blind opponents, irrespective of whether they themselves had no handicap or were disarmed (Fig. 3B). It seems rather that NO promotes the tendency to flee first in response to the opponent's actions. First, although blind crickets practically never lose against disarmed opponents (blind-Ringer, win chance: 87%, $n = 23$), they lost more than half of such contests when treated with SNAP (win chance: 35%, $n = 20$, χ^2 versus Ringer: 12.35, $P < 0.001$; Fig. 3A). Second, although disarmed contestants usually lose against blind opponents, they won almost half the fights when NO production was inhibited by LNAME (39%, $n = 31$, DNAME 8%, $n = 25$, $\chi^2 = 7.063$, $P = 0.008$). Hence, nitrergic drugs can compensate for the imposed handicaps, illustrating that NO translates information from the opponent's agonistic signals.

To test our hypothesis that crickets summate information from their opponent's actions during fighting for the decision to flee (12), we investigated how winners respond to new opponents at various times after victory. As in many species (18), crickets that win are typically highly aggressive (23) and, when matched 3 or 10 min after victory, fought
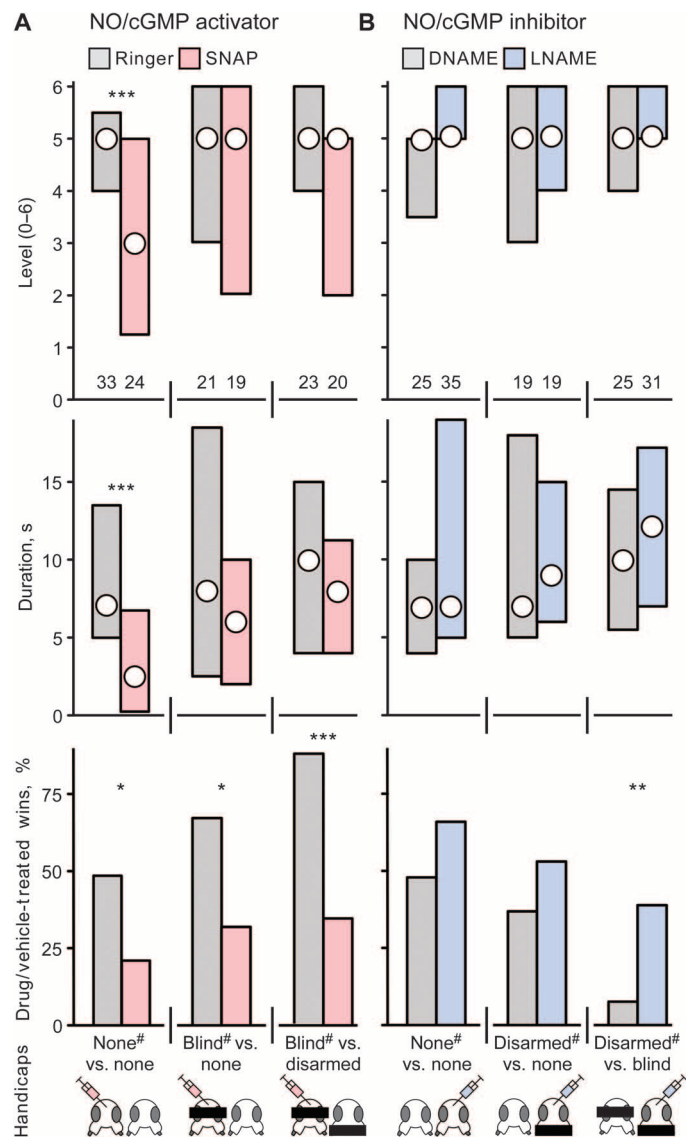
Fig. 3. NO translates the effect of the opponent's agonistic signals to promote the decision to flee. (**A**) Effect of the NO/cGMP pathway activator SNAP (red and gray bars: Ringer) on a contestant's aggressiveness when matched against opponents that received no drug (top, level; middle, duration; bottom, win chances; circles, median; bars, IQR; n is given on top x axis). As depicted from left to right, one or both contestants received either no further treatment or a handicap to impede transmission/perception of agonistic signals: None# versus none, Blind# versus none, Blind# versus disarmed (none, no handicap; blind, blackened eyes; disarmed, immobilized mandibles; # denotes drug/vehicle-treated contestant). (**B**) As for (A), but showing the effect of the NO/cGMP pathway inhibitor LNAME (blue bars) compared to its inactive enantiomer (DNAME, gray bars). Handicaps, from left to right: None# versus none, Disarmed# versus none, Disarmed# versus blind. Significant differences between drug-treated and control groups are indicated by asterisks (U test for level and duration, χ^2 test for win chances compared to controls: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Note that SNAP reduces the win chances of blind and LNAME increases win chances of disarmed, without any significant effect on escalation level and fight duration.

ferociously and won practically half the contests against standard hyper-aggressive opponents (Fig. 4; data for 10 min: median level 6, IQR 5 to 6, median duration 18 s, IQR 10 to 29, win chance: 47%, $n = 17$). However, when fights were staged immediately after victory, the winners were far less aggressive (U tests versus 10 min: $P_{level} = 0.0049$, $P_{duration} = 0.0014$), and they mostly retreated from hyper-aggressive opponents (16% wins, $n = 19$, χ^2 compared to 50%: 4.97, $P = 0.026$). This suggests that freshly established winners still bear a short-term record of their previous opponent's (the loser's) agonistic actions. Supporting this, and the notion that opponent actions activate the NO signaling pathway, the brief susceptible period when winners are more likely to lose was not evident in LNAME-treated winners (median level 6, IQR 4.75 to 6, median duration 17 s, IQR 7 to 22, win chance: 42%, $n = 26$, U tests versus DNAME: $P_{level} = 0.0077$, $P_{duration} = 0.0068$; Fig. 4). Blocking NO does not lead to a general increase in the tendency of winners to fight and win, because LNAME failed to increase the level and duration of fights or win chances against hyper-aggressive opponents when staged 3 or 10 min after the susceptible period.

To verify that sensory experiences are accumulated during fighting for the decision to flee, we tested the effect of potentially aversive stimuli on winner behavior. As shown in Fig. 5A, a single wind puff stimulus delivered to the abdominal cerci of a winner directly after victory resulted in a significant reduction in both the escalation level and fight duration in contests against hyper-aggressive opponents staged 10 min later (median level 3.5, IQR 1 to 5, $n = 20$, win chance: 20%, U tests versus winner that received no stimulus: $P_{level} = 0.0026$, $P_{duration} = 0.0075$; Fig. 5A). Of the 20 winners tested, 7 actually retreated on sighting the opponent. Two successive stimuli were even more effective and sufficient to convert practically all winners to behave like losers (median level 1, IQR 1 to 2.5, $n = 20$, win chance: 5%, U tests versus winner that received no stimulus: P_{level} and $P_{duration} < 0.0001$; Fig. 5A). This is not a general detrimental effect of aversive stimulation because it did not change winner performance when applied 3 or 10 min after the susceptible period (for example, for 3 min, U tests versus 0 min: P_{level} and $P_{duration} < 0.0001$; Fig. 5B). Furthermore, the effect is dependent on NO because it was not evident in LNAME-treated winners (median level 5, IQR 3.75 to 6, $n = 26$, U tests versus DNAME: P_{level} and $P_{duration} < 0.001$, 38% wins; Fig. 5C). LNAME did not, however, increase the aggressiveness of winners given the aversive stimuli after the susceptible period (3 and 10 min), illustrating again that LNAME does not cause a general increase in winner aggressiveness.

DISCUSSION

Our data reveal an intricate and key role for NO in controlling the expression of aggressive behavior in a model organism. We first showed that crickets treated with NO/cGMP pathway activators (SNAP and 8Br-cGMP) are less aggressive, whereas treatment with inhibitors (LNAME, PTIO, and ODQ) led to more aggressive and longer contests (Fig. 1). This contrasts an earlier study on socially naïve crickets with the same drugs, but with different analytical procedures, where no effects were resolved (17), but matches findings in mammals. For example, aggression increases in male rodents after knockdown of neuronal NOS (24, 25) or treatment with the neuronal NOS inhibitor 7-nitroindazole (26). However, despite recent advances linking NO to other transmitter systems affecting aggression, seasonal aggression, and human mental disorders [see (4, 13, 14) for reviews], its specific behavioral role during
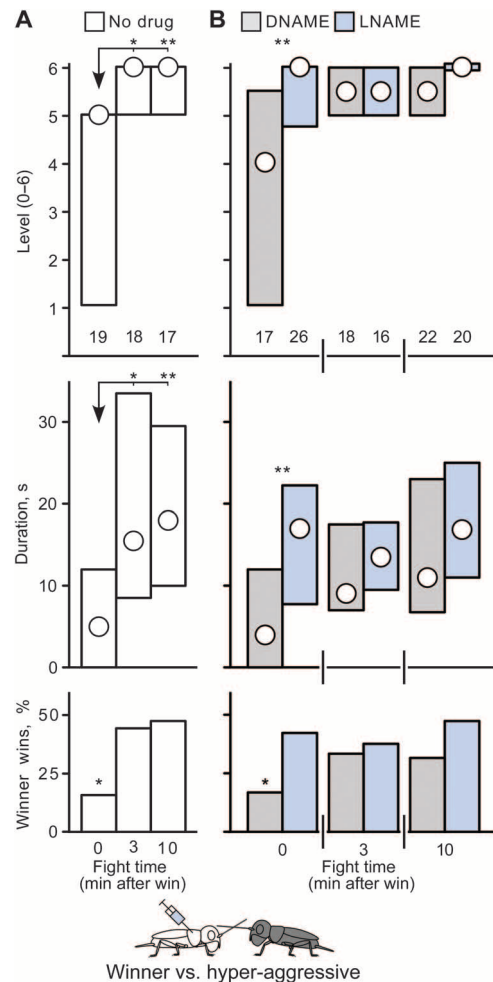
Fig. 4. The susceptible period in winners and its dependence on NO. (**A**) Bar charts showing the aggressiveness of untreated winners matched against standard hyper-aggressive opponents at different times after winning (top, level; middle, duration; bottom, win chances; different animals for each time slot). (**B**) As for (A) showing the effect of inhibiting NOS on winner performance against standard hyper-aggressive opponents (blue bars, LNAME; gray bars, DNAME). Significant differences are indicated by asterisks [level and duration in (A): U test, Bonferroni correction to α for three comparisons: *$P < 0.025$, **$P < 0.005$; in (B): U test: *$P < 0.05$, **$P < 0.01$; win chances: χ^2 test compared to 50%: *$P < 0.05$, **$P < 0.01$].

aggressive interactions is not clear. Our experiments on crickets reveal that NO is involved in the process of opponent assessment for the decision to flee and subsequent depression of aggression that follows social defeat in many animal species (18).

The decision to flee in crickets is based entirely on the opponent's agonistic signals perceived during fighting, which act to suppress aggression, so that individuals accumulating relatively more "punishment" will be the first to flee once a critical threshold is reached (12). In a key illustrative experiment, blind crickets practically always beat disarmed crickets because they perceive fewer agonistic signals than their opponents. Win chances are on par, however, when the blind crickets received an NO donor, or when NOS was blocked in their disarmed opponents (Fig. 3). These compensatory effects of nitridergic drugs
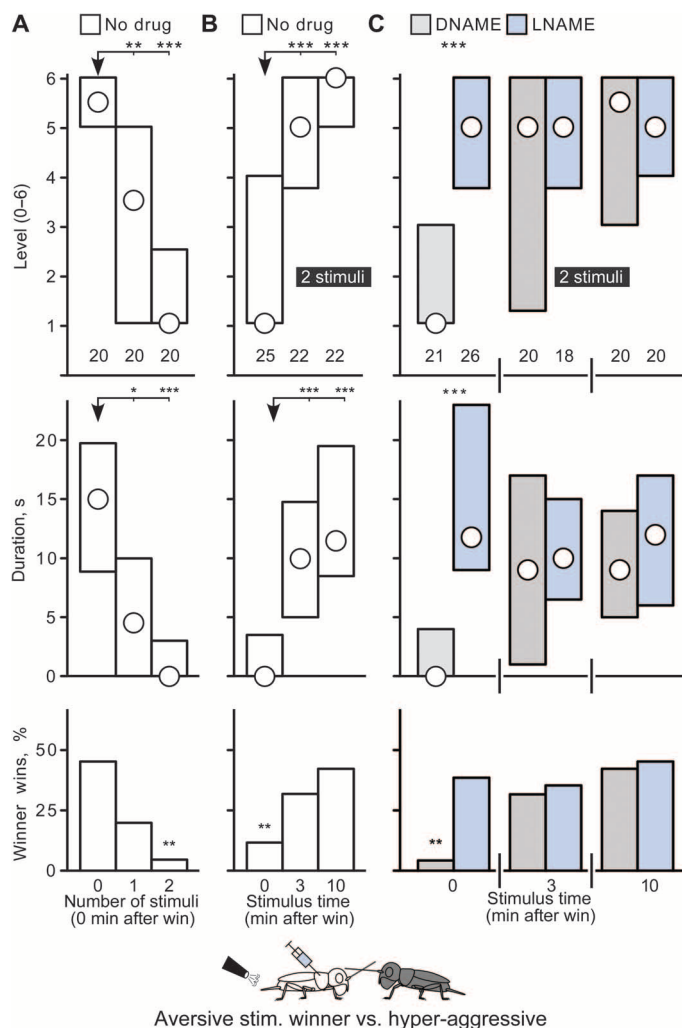
Fig. 5. Susceptibility of winners to aversive stimulation and its dependence on NO. (**A**) Bar charts showing aggressiveness of winners matched against standard hyper-aggressive opponents 10 min after a previous win that received either 0, 1, or 2 successive aversive stimuli (wind puff to cerci) during the susceptible period immediately after winning (different animals for each time slot). Top, level; middle, duration; bottom, win chances. (**B**) Bar charts, as in (A), showing the effect of two aversive stimuli delivered at 0, 3, or 10 min after winning. (**C**) Bar charts, as in (B), for winners that were given two aversive stimuli and treated with either LNAME (blue bars) or DNAME (gray bars). Significant differences are indicated by asterisks [level and duration in (A) and (B): U test, Bonferroni correction to α for three comparisons: *$P < 0.025$, **$P < 0.005$, ***$P < 0.0005$; in (C): U test, win chances: χ^2 test compared to 50%: **$P < 0.01$, ***$P < 0.001$]. Note that LNAME abolishes the effectiveness of the aversive stimulus to induce submissive behavior.

reveal that NO must somehow be involved in the sensory processing of agonistic signals. We therefore discount the possibility that these drugs influence aggression indirectly by affecting general physiological fitness. Supporting this, SNAP-treated crickets fight as harsh and as long as normal crickets against untreated opponents when blind (Fig. 3A). This finding also illustrates that NO is not acting to diminish an individual's tendency to escalate and invest time in fighting, that is, its aggressive motivation. Together, our data suggest that the sensory impact of the

opponent's agonistic actions leads to activation of the NO signaling pathway in the central nervous system, which in turn suppresses aggression by promoting the tendency to flee, rather than reducing the motivation to fight.

Supporting our hypothesis that competing crickets each add up their opponent's actions during fighting for the decision to flee, we found that winners exhibited a short period of susceptibility to attack just after scoring a win. Winners in most animal species are usually hyper-aggressive (*18*), as are crickets tested 3 to 20 min after victory (Fig. 4) (*23*). However, they mostly fled when rematched against new aggressive opponents immediately after their first fight (Fig. 4A). This is unlikely due to fatigue, which might be expected to last longer than the 3 min required to recover. Furthermore, crickets can fight for minutes without sign of physical exhaustion (*12*) and only minimal impact on energy expenditure (*27*). We suggest that this susceptible period represents the time span over which winners still bear a record of their previous opponent's actions. Supporting this idea, a short wind stimulus delivered during the susceptible period was sufficient to induce submissive behavior in a third of the winners, whereas two successive stimuli transformed almost all winners to behave like losers and retreat on sighting an opponent (Fig. 5A). It thus seems that winners, having previously approached the verge of losing, need to accumulate only a few more aversive experiences to become subordinate. Because the period of susceptibility to aversive stimuli in winners was not evident after inhibiting NOS with LNAME (Figs. 4B and 5B), it must be due to the action of NO. This rescue is not due to a general increase in aggressiveness because LNAME had no effect on winners when tested or stimulated after the susceptible period. We conclude that NO is a key component in the mechanism underlying the decision to flee in social conflict between male crickets. During fighting, each contestant adds up aversive stimuli derived from its opponent's agonistic actions. As soon as some critical amount has accumulated, this leads to activation of the NO signaling pathway, which promotes the decision to flee.

The susceptible period is also manifested in losers, only that in the absence of a countermanding winner effect [cf. (*23*)], it persists some 3 hours (*22*) and is referred to as post-conflict depression, or the loser effect, which accompanies social defeat in numerous species (*18*). As suggested by Iwasaki *et al.* (*17*), our data show that the loser effect in defeated crickets, just as the susceptible period in winners, results largely from activation of the NO signaling pathway: it was prolonged by the activators SNAP and 8Br-cGMP and shortened by the inhibitors LNAME, PTIO, and ODQ (Fig. 2). Again, NO need not suppress aggressive motivation because losers will fight fiercely, for example, when blind (*12*), suggesting that it renders losers more susceptible to aversive stimuli and hence more likely to flee on sighting an opponent.

The close association of NO-producing arbors with afferent fibers throughout the insect nervous system (*15, 28*) is aptly suited for integrating the net sensory impact of agonistic signals. Insect afferents are mostly cholinergic (*29*), and activation of nicotinic cholinergic receptors leads to NO/cGMP production (*30*), which is known to suppress neuronal activity (*31, 32*). NO is unlikely, however, to act on its own aggression. NOS colocalizes with numerous other neurotransmitters in insects (*33*), including γ-aminobutyric acid (GABA) (*34*), which mediates the acquisition of avoidance after social defeat in mice (*35*), whereas NO influences aggression in mammals by interacting with serotonin (*36*) and dopamine (*37*), both of which also modulate aggression in crickets (*38, 39*).

The challenge for the future will be to see whether the NO/cGMP pathway underlies the summing of information in other behaviors, such

as navigation and pathfinding in insects (40, 41), as well as the decision to flee and the loser effect in mammals. Social defeat is regarded as a major stressor, which plays a role in psychiatric disorders such as depression and post-traumatic stress disorder (42). We found that defeated animals, rather than being motivationally depressed, are more susceptible to aversive experiences and that the effect is also briefly manifested in winners (Fig. 4), where it is normally countermanded by the reward of winning (23). With the exception of an unusually high aggressive strain of *Drosophila* (43), this is the first time that normal dominant animals have been shown to be susceptible to attack after scoring a win. It will be intriguing to find whether the same applies to mammals, including humans. We now need to identify specific release sites and targets for NO in the cricket's central nervous system to unravel the neuronal circuitry and molecular mechanisms underlying its role in decision-making and social aggression.

MATERIALS AND METHODS

Experimental animals

Mature, 2- to 3-week-old, adult male Mediterranean field crickets, *Gryllus bimaculatus* (de Geer), were taken from a breeding stock maintained under constant standard conditions at Leipzig University (22° to 24°C, relative humidity 40 to 60%, 12-hour light/12-hour dark regime daily feeding on bran and fresh vegetables) and kept isolated in individual glass jars for at least 24 hours before the experiments. All experiments were performed during daylight hours, avoiding times when aggression tends to be depressed [just after midday and on generally dreary days, cf. (19)]. To further minimize random variations in daily performances, we tested single pairs of crickets from control and test groups (described below) in parallel and accumulated data from multiple daily sessions (maximally three groups per session, test sequence changed at each).

All animal treatments complied with the Principles of Laboratory Animal Care and the German Law on the Protection of Animals (*Deutsches Tierschutzgesetz*).

Evaluation of aggression

Aggressive behavior was evaluated in dyadic contests between equally sized males (<5% weight difference). The opponents were placed at opposite ends of a Perspex glass rectangular fighting arena ($l \times w \times h$: 16 x 9 x 7 cm) with a sand-covered floor divided halfway along its length by an opaque sliding door. On removing the door, the animals' interactions follow a stereotyped sequence typical for fights in the field, which we score on a scale of 0 to 6 to denote aggressive escalation (19) (details are given in the legend to fig. S1). Fight duration was measured to the nearest second with a stopwatch, deducting pauses that occasionally occurred when the animals lost contact. Fights were only evaluated when the outcome was clear in that the designated winner chased the loser, which retreated immediately. Win chances were evaluated when opponents received different treatments.

Pharmacological treatments

All drugs were obtained from Sigma-Aldrich; their sites of action are depicted in Fig. 1A. The most effective dosages and application method that changed aggressive behavior, but without any obvious detrimental effect on general motility, were determined in pilot investigations. Comparatively high concentrations are required due to the tight insect blood-brain barrier (21). In our experiments, test crickets were injected into the he-

mocoel via the pronotal shield using a microsyringe (Hamilton) with 10 μl of the following: vehicle—either Ringer for crickets (140 mM NaCl, 10 mM KCl, 7 mM CaCl$_2$, 8 mM NaHCO$_3$, 1 mM MgCl$_2$, 5 mM N-trismethyl-2-aminoethanesulfonic acid, 4 mM D-trehalose dihydrate) or DMSO (1% in Ringer); the NO donor SNAP (5 mM in Ringer); the membrane-permeable analog of 8Br-cGMP (1 mM in Ringer); the NO scavenger PTIO (10 mM in Ringer); the competitive NOS inhibitor LNAME (20 mM in Ringer) or its non-effective enantiomer DNAME (20 mM in Ringer) as an additional control; and the irreversible inhibitor of soluble guanylyl cyclase ODQ (1 mM in DMSO). Aggressive behavior was evaluated 30 to 60 min, maximally 4 hours, after pharmacological treatment. In some experiments, both opponents were drug-treated, and in others only one (stated in text).

Animal groups and physical treatments

To reveal mechanisms underlying opponent assessment during fighting, fights were staged with drug- and control-treated crickets after the following conditions, social experiences, and physical treatments.

Naïve. Crickets that had no social contact to conspecifics for 18 to 24 hours, after which all known effects of previous social interactions on aggressive behavior have abated (22).

Losers and winners. Losers are the first to retreat and normally avoid conspecific males for 1 to 3 hours after defeat ["loser effect"; (22)], whereas winners become highly aggressive and typically generate the rival song and body jerking movements ["winner effect"; (23)].

Blind. Crickets deprived of their opponents' visual agonistic signals by blackening their compound eyes and ocelli with enamel paint.

Disarmed. Crickets with mandibles immobilized by cutting the opener muscle tendon and hence reduced agonistic signaling ability.

Hyper-aggressive. Crickets that were flown tethered in a wind stream for 3 min, after which they become highly aggressive (21). They were used here as a near-standard hyper-aggressive opponent.

Aversive stimulation. A remotely controlled compressed air supply was used to deliver one to two air puffs (each 200 ms; 3 to 4 m/s, 1-s interval) from a 5-mm tube directed 5 cm from a cricket's abdominal cercal appendages. This served here as a standard aversive stimulus and has been used elsewhere to induce startle responses (21).

Data analysis

All statistical analyses were performed using standard commercial software (Prism 6, GraphPad Software Inc.) running on a Power Macintosh computer (Apple Computers). The median and the IQR were calculated for nonparametric data sets. The χ^2 test was performed to compare relative win frequencies, and the Mann-Whitney U test and the Wilcoxon signed-rank test were performed to test for significant differences in the distributions between unpaired and paired data sets, respectively. To avoid errors resulting from multiple comparisons, we routinely compared each test group with an individual control group. In some experiments, however, three groups were compared, so we applied the Bonferroni correction of α to avoid type I errors. The numbers of cricket fights for each experiment and test group are indicated in the figures.

SUPPLEMENTARY MATERIALS

Fig. S1. Stereotyped levels of escalating aggression in male crickets.
Fig. S2. Summary of effects of nitrergic drugs on loser recovery.

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Stevenson *et al. Sci. Adv.* 2015;1:e1500060   13 March 2015

7 of 7