Assessment of fight outcome is needed to activate socially driven transcriptional changes in the zebrafish brain

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Group living animals must be able to express different behavior profiles depending on their social status. Therefore, the same genotype may translate into different behavioral phenotypes through socially driven differential gene expression. However, how social information is translated into a neurogenomic response and what are the specific cues in a social interaction that signal a change in social status are questions that have remained unanswered. Here, we show for the first time, to our knowledge, that the switch between status-specific neurogenomic states relies on the assessment of fight outcome rather than just on self- or opponent-only assessment of fighting ability. For this purpose, we manipulated the perception of fight outcome in male zebrafish and measured its impact on the brain transcriptome using a zebrafish whole genome gene chip. Males fought either a real opponent, and a winner and a loser were identified, or their own image on a mirror, in which case, despite expressing aggressive behavior, males did not experience either a victory or a defeat. Massive changes in the brain transcriptome were observed in real opponent fighters, with losers displaying both a higher number of differentially expressed genes and of coexpressed gene modules than winners. In contrast, mirror fighters expressed a neurogenomic state similar to that of noninteracting fish. The genes that responded to fight outcome included immediate early genes and genes involved in neuroplasticity and epigenetic modifications. These results indicate that, even in cognitively simple organisms such as zebrafish, neurogenomic responses underlying changes in social status rely on mutual assessment of fighting ability.

social dominance | mutual assessment | fighting | gene expression | social genomics

Dominance hierarchies are ubiquitous in animal groups and play a key role in the regulation of social interactions between individuals competing for resources (e.g., potential mates), such that individuals of different social status commonly express different sets of behaviors (aka behavioral states) that match their competitive ability. Typically dominant individuals express competitive and resource monopolization behaviors (e.g., courtship behavior) that will potentially increase their Darwinian fitness, whereas subordinates refrain from direct competition for resources, thus avoiding costly social interactions (e.g., potential eviction from the group) in which they would have a low probability of success (1). However, this competition avoidance behavior of subordinates is only adaptive if it allows them to gain fitness advantages later on, for example, by taking over a vacant dominant role. Thus, it is important for subordinate individuals to be able to identify opportunities for social ascent and to rapidly switch their behavioral profiles accordingly (2, 3).

Despite the well-known genetic influences on aggressive behavior (4–6), social status depends to a great extent on group composition (i.e., relative competitive ability of group members) and on social factors, and the same individual must be able to switch between different social statuses (7, 8). Hence, the same genotype must accommodate the expression of multiple social phenotypes, and this should be accomplished, at least partially, by socially driven changes in gene expression in the brain that would lead to distinct transcriptome profiles across the social behavior neural network (aka neurogenomic states) (3, 9, 10) corresponding to status-specific behavioral states. Previous studies have established this mapping of socially dependent behavioral states onto neurogenomic states (11–14), and rapid responses to social interactions have also been described (15–18). However, the specific cue that signals changes in social status and triggers the switch between neurogenomic states has remained elusive. There are at least two potential cues of social status readily available in a social interaction: (*i*) the aggressive behavior expressed by the individual and (*ii*) the behavior expressed by the opponent. Animals may use either of these or a combination of the two to infer their social status (19, 20) and to trigger genomic and behavioral changes accordingly. For example, animals may only use self-assessment of their own behavior and trigger a dominant state above a certain threshold of expressed aggressiveness or any other self-measure of own competitive ability (21, 22); conversely, they may only assess the opponent's behavior and trigger the dominant state in response to observed submissiveness. Finally, animals may assess their relative competitive ability in comparison with the opponent by comparing their own behavior with that of the opponent. The two former scenarios are cognitively less demanding and

Significance

Within social groups, there are animals of different social status that express different behavioral profiles that are paralleled by different patterns of gene expression in the brain. However, social status is not fixed, but rather depends on social interactions; hence, group living animals must be able to switch between different status-dependent behavior and brain gene expression profiles. Here we show for the first time, to our knowledge, that what triggers a genomic response to a social interaction in zebrafish is the subjects' assessment of the interaction rather than a fixed response to a releaser cue in the environment. The occurrence of fighting assessment in zebrafish suggests that a cognitive ability classically considered complex is also present in a simple-minded vertebrate.

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provide a simple heuristic response, which is expected to be selected for rapid decision-making. In contrast, the latter scenario (i.e., mutual assessment) requires more complex cognitive abilities (i.e., the ability to compare two values of competitive ability: self vs. opponent) and should be slower but more accurate in terms of inference of relative competitive ability. Thus, the use of simpler or more complex assessment mechanisms can also be considered in light of the speed-accuracy tradeoff in decision making, which posits that decision quality depends both on its accuracy and on time to reach a decision (23). The current literature on fighting tactics in animals contests suggests that animals make the decision to give up fights based either on self-assessment or on opponent-only assessment of fighting ability, and evidence for mutual assessment of self vs. opponents' behaviors is scarce (19). However, the decision to shift social status given the cumulative experience of fighting success (or a single but highly salient fight experience) has broader phenotypic implications, because it often implies shifts in internal state (e.g., inhibition of reproduction in subordinates) and on behavioral profiles associated with social status. Therefore, triggering status-dependent changes in internal state and behavior are expected to rely on more accurate, albeit more delayed, assessment mechanisms such as mutual assessment.

In this study, we use zebrafish to test this hypothesis by manipulating their perception of fight outcome and assessing its effect on the brain transcriptome profile. For this purpose, we compare, using a genome-wide microarray gene chip, the neurogenomic response to social interactions between fish that fight a real opponent and fish that fight their own image on a mirror. Fish do not recognize themselves on a mirror and attack their own image as if it is an intruder (24). Mirror fights usually elicit similar levels of aggressive behavior to those of real opponent fights (25), but because submissive behavior is never expressed (i.e., the mirror image replicates the behavior of the focal fish), the former has no outcome, and the expression of aggressiveness is decoupled from the experience of winning or losing a fight. Size matched male zebrafish are socially isolated for 5 d before being exposed to a short-term (~30 min) social interaction that consists either in a mirror fight or in a real opponent fight. Aggressive behavior is quantified, and the identity of the winner and the loser of the real opponent fights are noted. A reference group remains in social isolation and do not experience any social interaction. Therefore, there are four phenotypes regarding social experience: mirror fighters; winners of a real opponent fight; losers of a real opponent fight; and socially isolated fish. These phenotypes differ among themselves in the combination of behavior expressed and behavior perceived in the opponent: winners express aggressive behavior and perceive submissive behavior in the opponent; losers expressed submissive behavior and perceive aggressive behavior in the opponent; and mirror fighters express aggressive behavior but also perceive aggressive behavior in the opponent. Therefore, the following predictions can be generated to identify which of the assessment modes described above better explains the neurogenomic response to social status: (*i*) if only the individuals own behavioral expression is relevant (i.e., pure self-assessment), then mirror fighters should have a response profile similar to that of winners; (*ii*) if only behavioral feedback from opponent is relevant (i.e., opponent-only assessment), then mirror fighters should have a response profile similar to that of losers; (*iii*) if the comparison between perceived behavior of the opponent with the expressed behavior is needed (i.e., mutual assessment), then mirror fighters should not activate a response because in mirror interactions they equal each other, and therefore no change in social status would be experienced by the subject.

Results

Behavior. Winners and mirror fighters expressed similar levels of aggressive behavior, whereas only losers expressed submissive behavior (Fig. 1). Due to the small sample size, no inference statistics were obtained and only descriptive data are presented. However, statistical validation of behavioral differences between the social treatments presented here in the same behavioral paradigm have been previously reported (25).

Differentially Expressed Genes. Contrasting each social treatment (i.e., winners, losers, or mirror fighters) with the reference group (i.e., isolated fish) revealed 168 differentially expressed (DE) genes across all treatments. Real opponent interactions elicited 151 DE genes in losers and 57 DE genes in winners, of which 40 were DE both in winners and losers (Fig. 2*A* and see Table S1 for complete list of DE genes). These socially regulated genes included neuronal activity-dependent immediate early genes (IEGs) [e.g., brain-derived neurotrophic factor (*bdnf*), B-cell translocation gene 2 (*btg2*), early growth response genes (*egr2a, egr2b, egr4*), FBJ osteosarcoma oncogene (*fos*), immediate early response 2 and 5 (*ier2, ier5*), jun B proto-oncogene (*junb*), neuronal PAS domain protein 4a (*npas4a*), nuclear receptor subfamily 4, group A, member 1 (*nr4a1*)], some of which involved in neural plasticity (e.g., *bdnf, btg2, egr2, egr4, junb, npas4, nr4a1*), and late genes playing a role in neural plasticity (e.g., *caprin1b*), neuronal proliferation and/or differentiation (e.g., *dusp5, gnb21l, hmx3*), association to neurological anomalies (e.g., *nras*), neurotransmitter transport (*slc6a19*), synapse function (e.g., *nlgn4a*), and neuronal dendrite extension and arborisation (*rundc3ab*) (Fig. 2*A*). Finally, there were also socially DE genes that interacted with histones and chromatin, thus having a potential role in epigenetic mechanisms [e.g., *epc1, jdp2, msl1b, msl2a, ncapd3, jade3,* Pim-1 proto-oncogene, serine/threonine kinase (*pim1*), *mnf40*]. Because previous studies have already documented the occurrence of DE genes between dominant and subordinate zebrafish, which included genes in the nonapeptides, serotonin, hypothalamo-pituitary-gonadal, and hypothalamo-pituitary-interrenal pathways (26, 27), we checked whether these genes were also responding to changes in status in our experiment. Interestingly, none of these genes were DE between winners and losers in our study (Table S2).

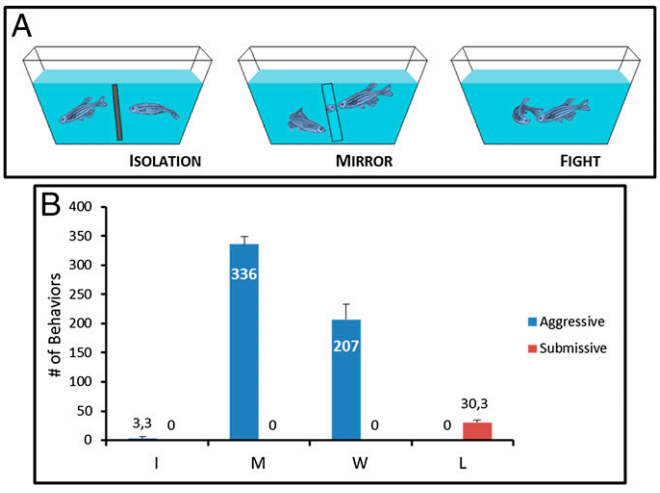

Fig. 1. Behavioral paradigm used to promote different social experiences in zebrafish. (*A*) Experimental setup used to promote the four social experiences: (*Left*) control group (no social interaction); (*Center*) mirror elicited fight (animals fought their own image on the mirror), and (*Right*) real opponent fights (animals fought a real opponent and experienced a victory or a defeat). (*B*) Behavioral profiles of each social phenotype (i.e., socially isolated, mirror fighters, winners, and losers) as illustrated by the frequency of aggressive and submissive behaviors (average ± SEM; $n = 3$ for each condition) expressed in the last 10 min of each type of social treatment.

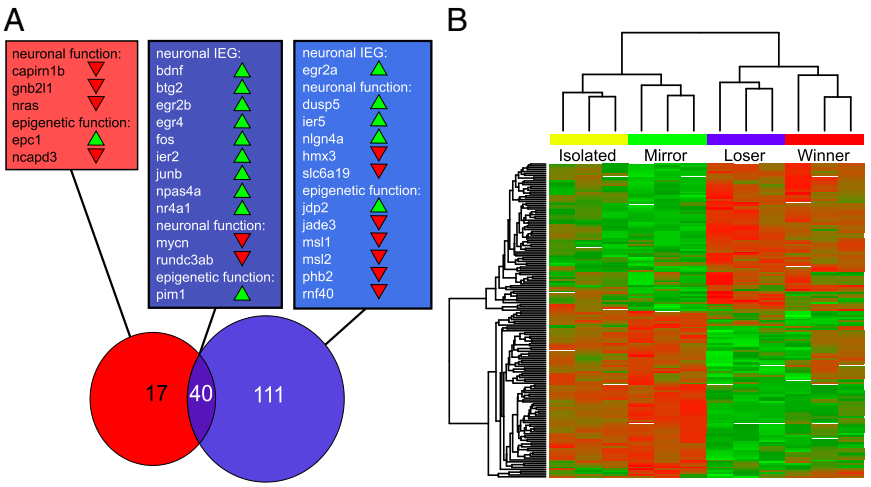

Fig. 2. Socially driven changes in gene expression in the brain of zebrafish. (*A*) Venn diagram showing the number of DE genes between each social experience (mirror fighting and winners and losers of a real opponent fight) and the reference group (social isolation). Only in winners (red) and losers (blue) were found DE genes. Genes with known effect in the neurosystem are also given (up-regulated = arrow up; down-regulated = arrow down). (*B*) Hierarchical clustering of the individuals from each social treatment (columns) and of DE genes (lines). Heatmap represents normalized gene expression levels (red, low expression; green, high expression).

In accordance with our hypothesis, mirror fights did not elicit any DE genes. This latter result should be interpreted with caution. Because we used a false discovery rate of 10% to control for false positives, the lack of response of mirror fighters at the transcriptome level does not mean that they would not show any differential gene expression if tested univariately (e.g., using a candidate gene approach), but rather that the fold change of putatively DE genes in mirror fighters was below the threshold for distinguishing them from baseline gene expression levels found in the reference group.

Hierarchical clustering of the samples indicated that all individuals from each social treatment were grouped together in individual clusters. Higher-order clusters subsequently grouped winners with losers and mirror-fighters with socially isolated individuals (Fig. 2*B*). This high consistency of the transcriptome profiles induced by each social experience indicates that the brain transcriptome of zebrafish closely reflects their recent acute social experiences.

Identification of Enriched Gene Ontology Processes, Molecular Pathways, and Chromosomes with Socially DE Genes. We tested for overrepresentation (ORA) of biological, molecular, and cellular processes in the DE genes of winners and losers. Gene Ontology (GO) analysis detected several biological processes enriched in both winners and losers related to neuronal activity, gene transcription, signaling, protein modification, development, and cell-faith regulation (Table S3). Molecular processes overrepresented in both winners and losers included terms related to gene expression (e.g., "DNA binding" and "Nucleic acid binding transcription factor activity") and potentially to epistasis (e.g., "Kinase activity" in winners; "Transferase activity," "Phosphatase activity" in losers). Losers also had enriched terms related to "Oxidoreductase" and "Signal transducer" activities (Table S3). Finally, regarding GO cellular component terms, both winners and losers seemed to have an overrepresentation of DE genes in the cell nucleus. ORA for pathways showed that both winners and losers have four enriched pathways in common (FGF signaling pathway; MAPK signaling pathway; oxidative stress; and ERK1–ERK2 MAPK cascade), whereas losers also have another enriched pathway, the TGF-β receptor signaling pathway (Table S3). ORA for chromosome location showed that losers have an enrichment of DE genes in chromosomes 9 and 14 with the proportion of up- and down-regulated being sensibly the same, whereas winners have an enrichment in chromosome 23 with almost all genes being up-regulated (Table S3).

Gene Modules Inferred by Weighted Gene Coexpression Network Analysis. The weighted gene coexpression network analysis (WGCNA) identified 12 coexpression gene modules, ranging in size from 54 to 1,969 genes (Fig. 3*A*). A 13th gene group (Gray) aggregated all of the remaining genes (628) that were not correlated with any of the coexpression gene modules. To assess the involvement of these gene modules in the social phenotypes (noninteracting, mirror fighters, winners, and losers) and observed behavioral traits (aggression and submission), associations between the eigengene of each gene module, which can be thought of as a weighted average module expression profile, and these phenotypes/traits were computed (using logistic regressions/correlations, respectively) (Fig. 3*A*). The four different social phenotypes were associated with different sets of gene modules, and only two modules were related to more than one social phenotype (i.e., noninteracting and loser; Fig. 3*A*). Mirror fighter and winners were associated with a single, but different, gene module: mirror fighters were characterized by an underexpression of the pale turquoise module (enriched in genes related to signal transduction, translation, and oxireductase and kinase activity, and in the pathway immune function signaling); Winners were characterized by a down-regulation of the dark-gray module (enriched in genes related to immune function, cell differentiation processes and peptidase and oxireductase activity, and in the pathway immune function signaling) (Fig. 3*B* and Table S4). In contrast, both noninteracting fish and losers were associated with various modules. Three modules were associated with losers: underexpression of the light yellow module (enriched in genes related to nitrogen compound metabolism, biosynthetic processes and RNA binding activity, and in the pathway cell cycle); underexpression of the dark green module (enriched in genes related to cell differentiation, nitrogen compound metabolism and histone binding, and in the pathway MAPK signaling); and an overexpression of the violet module (enriched in genes related to biosynthetic processes, nitrogen compound metabolism, and DNA binding, and in the pathway MAPK signaling) (Fig. 3*B* and Table S4). Noninteracting fish also had three modules associated with them: a unique down-regulation

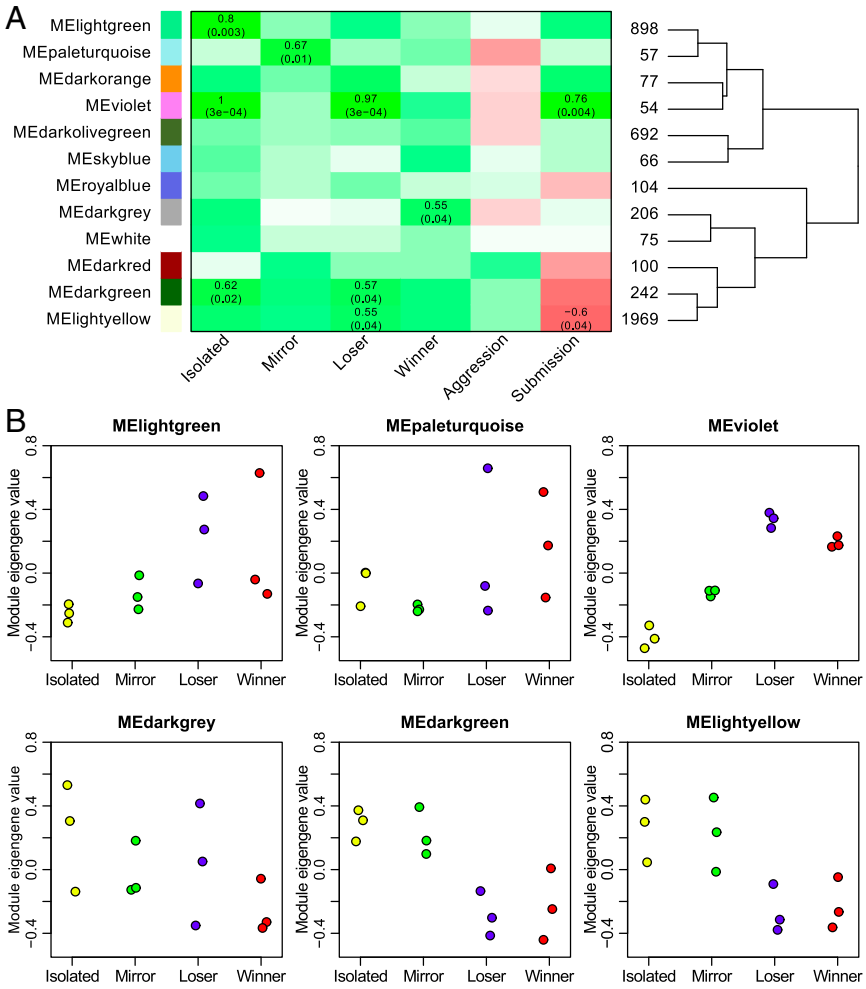

Fig. 3. Coexpression gene modules for different social experiences. (*A*) Associations between patterns of expression in the 12 identified modules across social phenotypes (noninteracting, mirror fighters, winners, and losers) and observed behavioral traits (aggression and submission). The colors of the boxes are scaled with the value of correlation coefficients, ranging from red (r = −1) to green (*r* = 1). On the right side of the heat map are the numbers of genes in each module and a dendrogram showing the inferred relationships among modules. (*B*) Eigengene values of samples separated by group (isolated, mirror, loser, and winner) for gene modules significantly associated to social phenotypes (light green, dark gray, pale turquoise, dark green, violet, and light yellow).

of the light green module (enriched in genes related to cell differentiation, biosynthetic processes and signal transduction, and in pathways neural crest development, BMP signaling, and fin development) (Fig. 3*B* and Table S4); an overexpression of the dark green module; and an underexpression of the violet module. Thus, in the two modules (dark green and violet) associated both with losers and noninteracting fish, these two phenotypes have opposing patterns of gene expression. Aggressive behavior was not significantly correlated with any of the gene modules, whereas submissive behavior was correlated with two modules (negatively with light yellow and positively with violet), which were also associated with the loser phenotype. These results suggest the involvement of different transcriptional networks in the observed social phenotypes.

We also checked whether the genes that have been reported to be DE between dominant and subordinate zebrafish in previous studies (26, 27) were overrepresented in the coexpression gene modules associated with the social phenotypes studied in our experiment (Table S2). Despite the presence of some DE genes from these previous studies in the coexpression gene modules reported here (*esr1* and *gnrh3* in the light yellow module associated with losers and *ar* and *esr2a* in the light green module associated with noninteracting individuals), none of these modules were overrepresented in the gene dataset of the previous studies (light yellow: OR = 0.17, $P = 0.99$; light green: OR = 0.50, $P = 0.90$).

Promoter Region Analysis. Promoter analysis identified transcription factor (TF) motifs associated with up- and down-regulated genes in winners and losers (Fig. S1) using cis-METALYSIS (28). Seven of the 13 enriched TF motifs were associated with gene expression changes in the same direction in both social treatments (i.e., 5 with up-regulation and 2 with down-regulation; Fig. S1*A*). Two other TF motifs, TFs *arnt* and *foxp2*, were associated with gene up-regulation only in losers. However, if we consider the enrichment of the interaction between pairs of TFs, both *arnt* and *foxp2* (Fig. S1 *B* and *C*) were also enriched in DE genes of both winners and losers. The remaining four enriched TF motifs, *junB*, *foxc1*, *hinfp*, and *max*, showed differential up- vs. down-regulation of DE genes between winners and losers. These latter results are not surprising because *junB, hinfp*, and *max* are known to act as either activators or repressors depending on context.

Discussion

A critical decision that group-living animals have to make is when to change social status given the available social information on

relative competitive ability, such that they avoid the costs associated with the expression of mismatched social status signals between their competitive ability and that of other group members (29). Here we tested the hypothesis that triggering status-dependent changes in internal state and behavior relies on assessment of fight outcome. We used the brain transcriptome as an inclusive phenotype, which reflects the breadth of neural and behavioral plasticity processes associated with status-specific organismal phenotypes. The results of this study support this hypothesis. Divergent changes in the brain transcriptome profile were observed between winners and losers of real opponent fights, which parallel the changes in behavioral state that have been described previously for zebrafish, such that winners of a single interaction significantly increase their probability of winning a subsequent interaction (winner effect), whereas losers decrease this probability (loser effect) (30). However, the transcriptome profile of mirror fighters, which lack information on fight outcome, was different from that of either winners or losers, despite expressing similar levels of aggressive behavior to that of winners of real opponent fights and being exposed to similar levels of aggressive behavior to that of losers of real opponent fights. Therefore, neither self-assessment (which predicts similar transcriptome responses between winners and mirror fighters) nor opponent-only assessment (which predicts similar transcriptome responses between losers and mirror fighters) can explain these results. Nevertheless, it can be argued that some components of the fight potentially critical for either self- or opponent-only assessment could be missing in mirror fights. To scrutinize this hypothesis, we analyze below the impact that two types of potentially relevant social information, which are missing in mirror fights, may have on our rationale. First, in mirror fights, the focal fish is not able to assume an antiparallel (i.e., head to tail) configuration with its opponent, which is commonly observed in early stages of real opponent fights when fish are lateral displaying to each other (31), and the mirror image never takes the initiative of displaying a different behavior from that expressed by the focal fish, which apparently leads to a lower frequency of displays but of longer durations (32). This slower pace of interactions with mirrors was not observed in zebrafish fights, where there was a similar frequency of aggressive acts between mirror fights and real opponent fights (Fig. 1*B*) (25). Moreover, the typical fight resolution time (i.e., the time it takes for a dominance-subordinate relationship to be established between a fighting pair) in real opponent fights is well below the fighting time used in the present study (30), and so there was enough time for either self- or opponent-only assessment to occur. Second, putative hydrodynamic signals from the opponent sensed by the lateral line (33, 34) are also absent in mirror fights, as well as potential physical injuries in escalated phases of the fight. However, a recent study has shown that in zebrafish visual information is sufficient for individuals to assess the social status of conspecifics and adjusts their behavior accordingly (35). Moreover, in our experimental setup, mirror fighters also had access to the holding water of other ongoing mirror fights, and therefore they were potentially exposed to any putative dominance chemical signals released in the water during the fights (e.g., dominance pheromone in cichlid fish) (36). Thus, the visual and chemical information available in mirror fights should be enough to convey the relevant information for dominance assessment. Moreover, the analysis of coexpressed gene modules (WGCNA) showed that the single gene module associated with mirror fighting was not related to either winners or losers and that winners and losers do not share any of the modules of coexpressed genes, which was an assumption for the testing of our hypothesis. Interestingly, there were no gene modules associated with the expression of aggressive behavior, which is in agreement with the observed differences between the neurogenomic states of winners and mirror fighters, which both express high levels of aggressive behavior. Together, the evidence discussed above rules out the proposed alternative explanations for our results and further supports the hypothesis that mutual assessment of fight outcome is needed to activate status-dependent transcriptomic responses.

The hierarchical clustering used in this study identified brain transcriptomic profiles that matched each of the social treatments: noninteracting, mirror fighting, winners, and losers. Also the WGCNA identified specific modules of coexpressed genes associated with each of the social treatments. Therefore, behavior-specific neurogenomic states seem to be present in zebrafish, and these reflect recent and acute social experiences. Although the two analyses presented in this study (i.e., DE genes analysis and WGCNA) use different approaches and reached different results in some aspects, overall they showed a high agreement in the characterization of the transcriptome changes associated with each social phenotype (Figs. 2*B* and 3*B*). Of the 168 DE genes, 83 were present in the gene modules identified by the WGCNA (Table S5). This number almost perfectly matches the prediction (84) because the WGCNA procedure filters half of the genes. Moreover, the representation of the DE genes in either winners (31/57 = 54.4%) or losers (77/151 = 51%) in the modules was also close to the expectations, indicating that this was not biased toward one of the phenotypes. Below we will integrate the results obtained using these two approaches for each phenotype (Table S5). First, despite the lack of single DE genes in mirror fighters, there was also a unique gene module associated with them (i.e., underexpression of pale turquoise module). This result is in agreement with previous studies that also found differences in immediate early gene expression in brain regions that belong to the social decision-making network in mirror fighters compared with noninteracting fish [zebrafish (37); cichlid fish, *Astatotilapia burtoni* (38)]. However, for all other gene modules with DE genes, mirror fighters showed levels of expression more similar to those of noninteracting fish than to those of either winners or losers (Fig. 3*B*). Thus, both analyses agree that despite also having a unique neurogenomic state mirror fighters are the interacting phenotype with the less differentiated brain gene expression profile from that of noninteracting fish. Second, except for the dark green module, noninteracting fish were characterized by an overall underexpression of the genes modules that comprise the DE genes. This result is not surprising considering that noninteracting fish were used as the reference phenotype for the DE gene analysis. In accordance with this view, only a small proportion of DE genes (nine DE genes in losers and one DE gene in winners) are present in the light green module that is uniquely associated with noninteracting fish. Third, none of the DE genes in winners were present in the dark gray module uniquely associated with them (by underexpression). In contrast, the genes identified as DE in winners appeared in different modules including the violet (14), dark green (12), light yellow (9), dark olive green (1) and light green (1). Interestingly, although the logistic regression of the WGCNA did not sort out the winners in these modules, for the first three modules, in which they had the largest number of DE genes, they had similar levels of expression to losers (Fig. 3*B*), which was the phenotype significantly associated with these modules (Fig. 3*A*). Thus, not only are most DE genes in winners also DE in losers (70.2%), but they also belong to gene modules associated with losers. These commonalities in socially driven changes in gene expression between winners and losers may thus reflect the engagement in a fight with a real opponent rather than status-specific triggered transcriptome profiles. These commonly DE genes between winners and losers include a large number of immediate early genes present in the violet (e.g., *fos, junb, egr2b, egr4, npas4, ier2, nr4a1*) and dark green (e.g., *bdnf, pim1*) modules, and some of these genes are involved in neural plasticity processes related to learning and memory [e.g., *bdnf*

(39); *npas4* (40, 41); *nr4a1* (42); genes of the *egr* family (43)]. Therefore, both winners and losers seem to be activating molecular pathways involved in memory formation, potentially related to previously described winner-loser effects in this species (30). This conclusion is further supported by the detected enrichment of the MAPK signaling pathway, which is also known to be involved in cognitive processes (44). The lack of similar activation patterns in mirror fighters may also suggest a failure of mirror fights to produce social memories. Fourth, there was a good match between the DE genes identified in Losers and the gene modules associated with them: of the 35 DE genes present in the light yellow module, 31 were DE in losers, and all of the DE genes present in the dark green and violet modules (12 and 22, respectively) were present in losers. There was also a small number of DE genes in losers that were present in modules not associated with them, such as the dark olive green (1) and the light green (9), and 2 DE genes in losers did not associate with any of the gene modules. Thus, 84.4% of the DE genes in losers that were present in the gene modules integrated modules significantly associated with them. Interestingly, losers and noninteracting fish lay at the extremes of expression of two gene modules (violet and dark green, with the former being overexpressed in losers and the latter in noninteracting fish) that comprise a number of immediate early genes and are enriched in the MAPK pathway. Together, these results suggest that different activity-dependent gene pathways are being differentially activated by the social interactions and that, among the three interacting phenotypes, losers are the ones that show the highest divergence from the noninteracting baseline. The higher expression of the dark green module in noninteracting fish and its association with histone binding also suggests that the 5 d of social isolation may have induced epigenetic changes in noninteracting fish, which are being reversed in interacting fish, in particular in losers (Fig. 3*B*). In agreement with this view, losers are the social phenotype with the highest number of DE genes involved in histone modification (i.e., histone H4 acetylation, histone H3 methylation, histone H2B ubiquitination; Dataset S1). Finally, the lack of association between aggression and any of the gene modules suggests that the neurogenomic state characteristic of either winners or mirror fighters is independent of their expression of aggressive behavior. In contrast, the neurogenomic state of losers is partially related to the expression of submissive behavior, because two of the three gene modules associated with losers were also associated with submissive behavior (the violet module positively and the light yellow negatively).

Overall our results show a higher impact of losing in comparison with winning in the neurogenomic state of the individuals, as indicated by both the number of DE genes and by the number of gene modules, which are three times higher in losers than that in winners (i.e., 151 vs. 57 DE genes and three vs. one associated gene modules). There are at least two possible explanations for these results. Social isolation has been suggested to induce dominance-like status in different species including fish (45, 46). If this is the case in zebrafish, than the behavioral state of winners is expected to be closer to that of socially isolated fish and that would explain the lower number of DE genes and gene modules in winners than losers, when using social isolation as a reference group. Alternatively, this result can be due to an asymmetry of the biological impact of winning vs. losing. In fact, the literature on winner-loser effects has shown that losing is more prevalent across different taxa and has a longer temporal expression (47), and in zebrafish, the magnitude of the loser effect in behavior is also much larger than that of the winner effect (30).

Our results differ from those of previous studies that had already established the occurrence of status-specific gene expression patterns in zebrafish, using a set of candidate genes covering different molecular pathways across a set of brain regions. These studies had found differential telencephalic and/or hypothalamic expression of nonapeptides, serotonin, hypothalamo-pituitary-gonadal, and hypothalamo-pituitary-interrenal genes (26, 27). In contrast, in the present study, when analyzed at the transcriptome level in whole brain samples, most of these genes for which a status-specific pattern was identified using a candidate gene approach were no longer detected as DE or overrepresented in the coexpression gene modules associated with specific social phenotypes. This discrepancy may result from methodological differences between the studies, namely the use of different interaction times (30 min vs. 1 d) and the different tissue coverage (whole brain vs. specific brain regions). For example, the simple fact that the profile of gene expression varies across brain regions may explain why whole brain data does not reflect regional patterns.

In summary, this study shows that assessment of fight outcome is needed to trigger status-specific neurogenomic states characterized by changes in gene expression and epigenetic markers in molecular pathways involved in neural plasticity processes underlying learning and memory. The broader implication of our results is that mutual assessment of competitive ability, which requires comparison of own vs. opponent fighting ability and hence has been considered as cognitively more demanding than either self-assessment or opponent-only assessment, may play a key role in social decision-making in "simple minded" animals, such as zebrafish.

Methods

Subjects and Maintenance. Zebrafish (*Danio rerio*) used in this experiment were WT (AB) acquired from the Zebrafish International Resource Center (ZIRC). Before the experiment, fish were kept in 40-L tanks (50 × 30 × 35 cm), in a 1:1 sex ratio, at 26 ± 2 °C and on a 14-h dark:10-h light photoperiod. Fish were fed twice a day with freshly hatched brine shrimp in the morning and commercial food flakes in the afternoon. Average fish size was 27.1 ± 1.7 mm (standard length). The animal experimentation procedures used in this study were approved by the internal Ethics Committee of the Gulbenkian Institute of Science and by the National Veterinary Authority (Direção Geral de Alimentação e Veterinária, Portugal; permit no. 8954).

Behavioral Assays. We used a modified version of an isolation-induced aggression paradigm, which is known to promote the expression of aggressive behavior (30). In brief, fish were isolated 5 d before the social interaction. To test for the effects of the interaction outcome, two other groups were used: a social isolation group and a mirror elicited aggression group. Twenty-four adult males, matched for standard length (size difference < 2 mm), were exposed to one of four experimental social experiences: winning the interaction (winners), losing the interaction (losers), an unsolved interaction (mirror fight), or experience no interaction (reference group, isolation). We assumed that the social isolation period was enough to extinguish previous social experience (i.e., to reset social status) and focal fish were considered neutral in terms of social status (i.e., neither dominant nor subordinate). Therefore, winners can be seen as gaining social status (i.e., becoming dominant) and losers as losing social status (i.e., becoming subordinate). However, one can dispute that the social status of participants at the start of the social interactions was neutral. In fact, two alternative scenarios are possible: (*i*) if one assumes that during social isolation, in the absence of competition, males became territorial (and thus they should be considered dominant), then the change in social status is differential between winners, which only reinforce their already dominant status, and losers, which switch from dominant to subordinate; and (*ii*) if one assumes that, due to social stress imposed by social isolation, males become subordinate during this period, then the change in social status is also differential between winners, which switch from subordinate to dominant and losers, which only reinforce their subordinate status. It is important to stress that all these assumptions involve changes, of varying magnitude, in social status and that given the use of the socially isolated treatment as a reference group, all experimental groups are being tested against whatever the social status at the start of the interactions was.

Fish were always tested in pairs to control for spurious effects of putative chemical communication that would otherwise only be present in fighting dyads. Each pair was placed in a 700-mL polycarbonate breeding tank

(18 × 10 × 9 mm) isolated visually, but not chemically, by a removable opaque PVC partition for 5 consecutive days. Therefore, if putative chemical cues are released by fighting males, they will also become available to individuals of each pair of mirror fighters. After this period, the opaque divider was removed in all conditions, which allowed contact between the two conspecifics in the fighting dyads; contact with the mirror in the mirror fighting treatment; and control of stress induced just by the movement of the partition in the isolation group. In the real opponent treatment, fight duration was set to 15 min after the interaction was solved (i.e., a clear winner and loser phenotype emerged). Given that fight resolution time varied from interaction to interaction, average total interaction time in real opponent fights was 36.3 ± 3.6 min (mean ± SEM). The duration of the other social treatments (mirror; isolation) was thus set to 30 min, such that all social treatments had a similar duration.

Behavioral Analysis. Video recordings (Sony KDL X200) were analyzed using the software Observer XT (Noldus). An experienced observer analyzed the behavioral interactions according to the zebrafish ethogram (30). Behaviors were divided into aggressive (bite, chase, strike) and submissive (freeze and flee). Because we were only interested in the behavioral output resulting from social interactions, we only analyzed the postresolution phase of the fight, where different social phenotypes (winners, losers) can be clearly identified. For the behavioral analysis of mirror fights and social isolation, the last 10 min of the behavioral trial were also observed.

Tissue Processing, RNA Extraction, and Gene Expression. Immediately after the social interactions, fish were killed with a lethal dose of MS-222 (1,000–1,500 mg/L) and decapitated. Brains were rapidly collected in 500 μL Quiazol (Qiagen) and stored at −80 °C until further processing. Total RNA was extracted according to the manufacturer's instructions (RNeasy Lipid Tissue Mini Kit; Qiagen). RNA was then treated with DNase (RNase-free DNase set; Qiagen) to remove possible contaminations with genomic DNA, and concentration and purity were estimated by spectrophotometric absorbance in a NanoDrop ND-1000 UV-Vis Spectrophotometer (Nano-Drop Technologies). Total extracted RNA was kept at −80 °C until processing.

Target Synthesis and Hybridization to Affymetrix GeneChips. RNA was processed for use on Affymetrix GeneChip Zebrafish Genome Arrays, according to the manufacturer's GeneChip 3′ IVT Express kit user's manual. In brief, 100 ng total RNA containing spiked in Poly-A RNA controls was used in a reverse transcription reaction (GeneChip 3′ IVT Express Kit; Affymetrix) to generate first-strand cDNA. After second-strand synthesis, double-stranded cDNA was used in a 16-h in vitro transcription reaction to generate aRNA (GeneChip 3′ IVT Express Kit; Affymetrix). Size distribution of the aRNA and fragmented aRNA, respectively, was assessed using an Agilent 2100 Bioanalyzer with a RNA 6000 Nano Assay; 15 μg fragmented aRNA was used in a 250-μL hybridization mixture containing added hybridization controls. Two hundred microliters of mixture was hybridized on arrays for 16 h at 45 °C. Standard posthybridization wash and double-stain protocols (FS450_0004; GeneChip HWS kit; Affymetrix) were used on an Affymetrix GeneChip Fluidics Station 450. Arrays were scanned on an Affymetrix GeneChip Scanner 3000 7G.

Gene Expression Analysis. Scanned arrays were analyzed first with Affymetrix Expression Console software to obtain Absent/Present calls and to assure that all quality parameters were in the recommended range. Subsequent analysis was carried out with Partek Genomics Suite v. 6.6 (Partek Incorporated). After performing a standard RMA normalization, a two-way ANOVA ($P < 0.01$) was used to identify genes in the target groups (winners, losers, or mirror) differently expressed from the reference (isolation) taking into account batch effects (i.e., date of the microarray processing) and social treatment. Multiple testing was corrected using a false discovery rate of 10% and a minimal fold change of 1.1. Using gene expressions, hierarchical clustering of both samples and genes was calculated using Euclidean distances and average linkage.

Annotation and Gene Ontology Analysis. Genes were annotated using Entrez IDs obtained primarily from the Bioconductor database, National Center for Biotechnology Information (NCBI), and biomart. A total of 10,488 genes were annotated, from which 9,725 had information on chromosome location. ORA were performed to assess if the DE genes of each social treatment (winners and losers) were enriched in some gene set. The gene sets considered were terms from GO, pathways from Wikipathway, and chromosome locations (Table S3). Gene sets with less than three genes were discarded, and the threshold for overrepresentation was set to $P < 0.05$. These analyses were performed using Bioconductor packages "zebrafish.db," "GO.db," "biomaRt," "reutils," and "GOstats."

Gene Coexpression Network Analysis. WGCNA was used to find clusters of coexpressed genes (48). Each gene module-weighted average expression profile was summarized in an eigengene. Correlations between the eigengene of each gene module and the social phenotypes (noninteracting, mirror fighters, winners, and losers) and observed behavioral traits (aggression and submission) were computed to assess the involvement of each module on each social phenotype/behavior. Gene modules were characterized using ORA for gene ontology, pathways, and chromosomes, as described for the analyses on the DE genes.

Promoter Region Analysis. TF binding sites (motifs) were searched in upstream regions of the zebrafish genome by calculating scores using Stubb 2.1 (49). These scores were used to perform enrichment analysis using cis-METALYSIS (28) by considering the sets of DE genes identified for each social treatments (winners and losers). The algorithm used for these analyses is similar to the procedure by Sanogo and coworkers (17) and is detailed in *SI Methods*. In brief, genomic information was obtained from the University of California Santa Cruz Genome Browser, to which Stubb was used to score motifs every 500-bp window with a 250-bp shift. Nonredundant motifs from the Jaspar Core Vertebrate database (50) were considered. Enrichment analysis was then performed for each motif and pair of motifs using cis-METALYSIS (mode "flexible"). Analyses were performed using the mentioned software within a python pipeline (scripts available on request).

Confirmatory Real-Time PCR. To validate the microarray data, the expression of DE genes with higher fold changes was independently quantified by quantitative RT-PCR. All tested genes yielded similar patterns of relative expression across treatments as the ones obtained from microarray data (Table S6 and Fig. S2).

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