Developmental Glucocorticoid exposure in zebrafish alters adult behavior and primes differential expression of genes linked to human psychiatric disorders

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Abstract

Chronic or severe stress during development increases psychiatric disease susceptibility. One putative mechanism involves priming of stress-sensitive gene networks by early life Glucocorticoids (GCs) exposure, which modifies responses to stress later in life. However, brain-wide GC-primed molecular alterations are currently unknown. Here, we used an optogenetic zebrafish model to increase endogenous GCs during development and identified comprehensive transcriptomic alterations in the whole brain of GC-exposed fish across the life course. When exposed to acute stress in adulthood, developmental GC-exposed animals show highly exaggerated and pervasive brain-wide transcriptional changes which include gene sets associated with axon development, neuronal signaling, and epigenetic modulators. Strikingly, these altered gene sets showed an overrepresentation of human psychiatric disorder risk genes. We identify in developmental GC-exposed individuals: 1) altered biological processes, 2) hitherto uncharacterized novel GC-primed genes, 3) altered adult behaviors, and 4) subsets of primed genes and putative transcriptional regulators associated with changes in adult social behavior including oxytocin and *myt1la*. Thus, our study establishes a new translationally relevant zebrafish model and suggests potential molecular mechanisms by which developmental GC-exposure exerts its life-long impact.

Introduction

Early life stress (ELS) can lead to long-term alterations in neural plasticity, functional connectivity, epigenetic dynamics, and behavior[1-4]. Reflecting these far-reaching effects, ELS is a key factor affecting healthy development and aging as well as susceptibility to psychiatric disorders in humans[5-8]. The molecular response to stress in vertebrates is primarily mediated by a highly conserved neuroendocrine stress regulatory system called the Hypothalamo-Pituitary-Adrenal axis (HPA-axis) in humans[9] and the Hypothalamic-Pituitary-Interrenal axis (HPI-axis) in fish[10]. Like the mammalian adrenal gland, the fish interrenal gland is composed of two cell types: aminergic chromaffin cells and steroidogenic interrenal cells, the latter of which produce glucocorticoids (GCs), effector stress hormones that are highly conserved across the vertebrate kingdom[9]. While GCs have pleiotropic effects on many aspects of animal physiology, exposure to elevated GCs during brain development is known to cause long-term alteration of behavior, physiology, and stress regulation[1]. However, how high GC exposure during development predisposes individuals to adulthood dysfunction is poorly understood. One potential mechanism is long-lasting epigenetic changes, which modify how animals respond to stress in adulthood. For example, altered DNA methylation at

the glucocorticoid receptor (GR) gene promoter by poor maternal care is associated with adulthood alterations in histone acetylation, DNA methylation, transcription factor binding, GR expression, and HPA response to stress[11]. Moreover, a recent study identified differentially expressed genes (DEGs) "primed" by ELS in specific regions of ELS-exposed mouse brains[12]. These genes showed enhanced differential expression in adulthood upon re-exposure to stress and may be associated with behavioral changes in adulthood. Another study identified GC-primed molecular alterations using a GC (dexamethasone)-exposed human hippocampal progenitor cell line and identified long-lasting DNA methylation alterations[13]. Lastly, genetic differences in the immediate transcriptome response to GR activation are shown to predict risk for depression and co-heritable psychiatric diseases[14]. Together, the above studies establish that ELS or developmental GC exposure results in distinct responses to stress later in life by priming and maintaining longer-term alteration of specific genes which facilitate increased psychiatric disease susceptibility. However, so far, ELS or GC-primed genes have only been identified in specific regions of the brain including reward circuitry or cell lines, and brain-wide GC-primed molecular alterations have not been investigated.

In this study, we utilized optogenetic manipulation of steroidogenic interrenal cells in a zebrafish model to increase the endogenous GC level during larval and juvenile stages. Although the mammalian counterpart of the fish interrenal, the adrenal gland, is known to produce three types of steroid hormones, GCs, mineralocorticoids (MCs), and androgens[15], MCs and androgens are not detectable in larval zebrafish interrenal gland[16-19]. Therefore, our manipulation is suitable to assess specific effects of elevating endogenous GCs. As a first step in determining the effects of developmental GC exposure, the zebrafish were exposed to high levels of GCs throughout development starting from the postembryonic stage.

Here we report brain-wide transcriptomic alteration of GC-exposed fish across the life course. Strikingly, when subjected to acute stress in adulthood, developmental GC-exposed fish show highly exaggerated endocrine and transcriptional responses, indicating that adult stress is processed differently depending on GC exposure history. Remarkably, the genes primed by GC-exposure were enriched in gene sets associated with human neuropsychiatric disorders indicating high levels of evolutionary conservation in how early GC exposure leads to adult dysfunction. We further identified 1) biological processes altered by developmental GC exposure, 2) adult behaviors altered by developmental GC exposure, 3) hitherto uncharacterized novel GC-primed genes, and 4) subsets of primed genes and putative

transcriptional regulators associated with changes in adult social behavior induced by developmental GC exposure including oxytocin and *myt1la*.

Methods and materials

The detailed protocol for all the procedures used in this study is available as step-wise online methods[20].

Zebrafish husbandry and maintenance

The tübingen (TU) strain was kept at 28□ on a 12:12 hour light/dark cycle and housed at a maximum density of 5 fish/L. For experiments with adult fish, only females were used. All animal procedures were carried out in compliance with the ethical guidelines of the national animal welfare law and approved by relevant authorities (Landesuntersuchungsamt Rheinland-Pfalz, Germany, Project number 23 177-07/G20-1-033 or UK Home Office PPL number PEF291C4D).

Generation of transgenic line

Linearized pT2-2kbStAR:bPAC-2A-tdTomato[21] (ZFIN ID: ZDB-GENE-001120-3) recombinant plasmids were injected into 1-cell stage wild-type embryos of TU strain at 10-50 ng/mL in the presence of 10-50 ng/mL Tol2 transpose mRNA and 0.05% phenol red. The progenies of injected fish were screened for interrenal-specific tdTomato expression and the stable transgenic line, *Tg(star:bPAC-2A-tdTomato)*^{uex300} was established.

Sample collection

All samples were collected within a 2-hour window in the morning (08:30 to 10:30) without feeding.

Whole-body Cortisol assay

The competitive cortisol assay (Cisbio HTRF® Cortisol Kit, 62CRTPEG) was performed following the manufacturer's protocol. ELISA signal was detected by CLARIO star plate reader (BMG Labtech, Ortenberg, Germany).

Behavior tests

Social behavior tests and acute stressor delivery using looming dot (LD) presentation were carried out as described in Cook *et al*.[22]. Basal locomotion, food consumption and fear

conditioning assay were performed following the protocol in online methods[20]. For all behavioral tests, female adult zebrafish (6-9 months old) were moved into a designated behavior experimental room one week prior to testing and housed in groups of 10 fish in 3L tanks. Experiments were carried out between 09:00 and 14:00 and fish were not fed in the morning of testing. Fish were not used in any repeat experiments. The trials were recorded at 30 FPS. A camera (acA1300-60gm, Basler ace) was positioned at the front and/or top side of the tank. The test fish was tracked using Ethovision (EthoVision XT 13, Noldus Information Technology).

RNA preparation and quantification

Each sample for the larval stage (6 and 13 dpf) contained 25-30 larval whole brains whilst for the adult stage (120 dpf) three brains constituted one sample. Transcripts were quantified using real-time qPCR and next-generation mRNA sequencing. Primer information for qPCR is described in Supplementary Table 1. A total of 60 samples was sequenced, consisting of 5 biological replicates at four different time points (6, 13, 120 dpf, and acute-stressed at 120 dpf) for each genotype (wild type, bPAC+ and bPAC-).

Immunohistochemistry

Immunohistochemistry was performed as described with minor modifications[23]. Custom-made primary rabbit anti-oxt antibodies (1:200 dilution)[23] and Alexa 488 anti-rabbit antibodies (A-11008, Invitrogen, USA) as secondary antibodies (1:1000 dilution) were used for the visualization of oxytocinergic neurons.

Imaging and analysis

Confocal stacks through the larval head were recorded using a Zeiss LSM 880 with Airyscan FAST with PApo 10X/0.45 – dry objective with Airyscan mode. Stacks were analyzed using ImageJ (FIJI, 1.53c)[24]. Corrected Total Cell Fluorescence (CTCF) was calculated as integrated density − (Area of cells × Mean fluorescence of background reading).

Statistics

Statistical analyses were performed using R and Prism 9 (Graphpad Software Inc, San Diego, CA, USA). A detailed description of the statistical analyses is provided in online methods[20]

Data availability

All sequenced reads for RNA-seq were deposited in European Nucleotide Archive (ENA, PRJEB53713).

Code availability

The detailed data processing and R scripts for bioinformatic analysis are available at the GitHub repository (https://github.com/minkechoi/tx_star-bPAC_brain).

Results

Optogenetic elevation of endogenous GC during development

We have previously reported that elevation of endogenous cortisol (GC in fish) is induced in a transgenic zebrafish line by blue light present in ambient light which activates *beggiatoa* Photoactivated Adenylate Cyclase (bPAC), expressed specifically in steroidogenic interrenal cells[21, 25]. For the present study, we generated a new transgenic line, *Tg(star:bPAC-2A-tdTomato)*^{uex300}, using the same construct in a different strain to obtain a higher cortisol level than what was previously feasible and raised the fish under ambient light (hitherto referred to as bPAC+) (Fig. 1a,b). Indeed, we observed a strong cortisol increase at 5 days post fertilization (dpf) in bPAC+ compared to bPAC-, siblings of bPAC+ which do not carry the transgene (Fig. 1c). bPAC+ larvae raised under a filter, which restricts blue light penetration, did not show a cortisol increase, supporting that the cortisol elevation was due to optogenetic activation of bPAC (Fig. 1c). Moreover, the expression of *fkbp5*, which regulates the responsiveness of the glucocorticoid receptor (GR)[26], was increased in bPAC+ brains compared to bPAC- and to wild type, confirming the activation of the GR signaling cascade (Fig. 1d). Although bPAC- fish did not carry the transgene, since transgenerational inheritance of stress-induced phenotypes is reported in mammals and fish[27-29], we compared bPAC+ and bPAC- to each other, as well as to wild type in this study. Consistent with the reported onset of endogenous *star* gene expression[30], an increased cortisol level was observed starting around 2 dpf (Fig. 1e). The high cortisol level was sustained during larval and juvenile stages in bPAC+ fish. However, by adulthood (three months post-fertilization (mpf)), no significant difference was detected between bPAC+ and wild type (Figs. 1e,f). These results indicate that by using bPAC+ we can achieve a robust and persistent increase in endogenous cortisol levels throughout development.

Alteration of adult behavior in developmental GC-exposed fish

We assessed whether bPAC+ fish exhibited behavioral changes as adults using a set of tests aimed at assessing behaviors commonly affected in neuropsychiatric diseases[31]. We first established that bPAC+ fish did not show significant differences in basal locomotor activity compared to wild-type and bPAC- fish (Figs. 2a,b). We then focused on social behavior as social processes are translatable across multiple species and are often perturbed in a range of disorders associated with ELS[32]. To assess social behavior, we employed a new test that we recently established, which allows the assessment of distinct phases of social behavior, namely a drive to approach a social cue, and an ability to engage and maintain social interaction (Fig. 2c)[22]. In this test, after the fish was placed in a test tank and before the presentation of conspecifics, wild type, bPAC- and bPAC+ all displayed preference for the blue-colored bottom zone with no significant genotype differences (Figs. 2d,e). When the social cue was revealed, within 10 seconds the vast majority of wild type, bPAC-, and bPAC+ entered the social interaction zone (shaded green in Fig. 2f) adjacent to the conspecifics, within which wild types display a stereotypical C-shaped pattern of swimming (Fig. 2h). No difference in social approach between the groups was found using a Log-rank test (P=0.77), indicating that bPAC+ did not show deficits in recognition nor approach to social cues (Fig. 2g). In contrast, we found significant differences between wild type and bPAC+ during the 2-minute social phase in the time spent in the interaction zone and the number of social interactions performed (Figs. 2h-j). bPAC- also spent less time in the interaction zone and performed fewer interactions than the wild type (Figs. 2i,j).

Next, we tested food consummatory behavior by counting the number of floating food pellets eaten by a fish during a 10-minute interval (Supplementary Fig. 1a). Wild-type adult fish consumed on average 14.5 pellets while bPAC+ fish consumed on average 2.33 pellets out of a total of 25 (Supplementary Figs. 1b,c). This was not due to the lack of recognition nor interest in the food, as bPAC+ fish approached the food pellets and actually spent a longer time in the zone near the pellets (feeding zone) than wild types (Supplementary Fig. 1d).

To assess cognitive processes, we tested associative learning using a Pavlovian avoidance learning test where fish learn to associate the color green with a mild electric shock in the testing chamber (Supplementary Figs. 1e,f). Fish were first habituated in the chamber for 30 minutes and tracked to determine the baseline preference for green or blue color. Wild-type and bPAC+ fish showed no significant differences in baseline color preference (Supplementary Fig. 1g). However, after the conditioning, wild-type fish spent on average 89.2% of their time in the blue zone, significantly more than bPAC+ fish who spent on

average 62.68% of their time in the blue zone (Supplementary Fig. 1h), suggesting a deficit in associative learning in bPAC+ fish.

Lastly, we tested the behavior of bPAC+ fish in a novel environment using a novel tank test (Supplementary Figs. 1i-m). In the novel tank test, wild-type fish first dive to the bottom and demonstrate a sustained depth-preference whilst performing bouts of erratic high-speed swimming[33, 34]. In contrast, bPAC+ fish showed reduced depth-preference (Supplementary Fig. 1j) and generally swam slower than wild-type controls (Supplementary Fig. 1k). Time spent performing fast-swimming bouts was also significantly decreased in bPAC+ fish (Supplementary Fig. 1l). However, there was no significant difference in freezing duration between groups (Supplementary Fig. 1m).

Together these results show that social interaction, food consumption, associative learning, and adaptation to a novel environment are altered in bPAC+ fish compared to wild types. Our results point to modification of a broad range of goal-directed behavior performance without changes in basal locomotion parameters in bPAC+.

Whole-brain transcriptional alterations in bPAC+ fish across the life course

Whole-brain RNA sequencing was carried out using larval (6 and 13 dpf) and adult (120 dpf) brains to identify and compare transcriptomic differences in wild type, bPAC+, and bPAC-. A large number of DEGs was identified in pairwise comparisons of bPAC+ and wild-type brains at 6 dpf (up/down=602/1253), 13 dpf (up/down=777/1566), and 120 dpf (up/down=684/1078) (Fig. 3, Supplementary Table 2). At all-time points, the number of downregulated genes was greater than the number of upregulated genes, indicating that developmental GC-exposure tends to repress rather than activate genes (Fig. 3a). Further, the number of DEGs consistently downregulated from early life until adulthood (273 DEGs) was greater than the upregulated DEGs (48 DEGs) (Fig. 3a). We identified fewer DEGs when comparing bPAC- to wild type, with 571 (up/down=329/242), 1026 (up/down=372/654), and 74 (up/down=42/32) DEGs at 6, 13, and 120 dpf, respectively, and many of these DEGs overlapped with those identified in the bPAC+/wild type comparison (Supplementary Fig. 2a). To compare the overall transcriptomic landscape of all identified DEGs, we performed unsupervised hierarchical clustering, which revealed large differences in the gene expression landscape between the bPAC+ and bPAC- compared to wild type (Fig. 3b). At each time point, bPAC+ and bPAC- transcriptome profiles clustered together separately from those of wild type. Next, to confirm an increase in GR signaling, we measured the expression level of GR target genes, *nr3c1*, *nr3c2*, *fkbp5*, *tsc22d3*, and *hsd11b2* at 6, 13, and 120 dpf. The

expression levels of *nr3c1, fkbp5, tsc22d3,* and *hsd11b2* were altered at multiple time points in bPAC+ (Fig. 3c). bPAC- also showed differences to wild type, albeit generally to a lesser degree (Fig. 3c). Both the alteration of GR signaling and similarity of DEGs identified in bPAC- and bPAC+ point to potential transgenerational effects present in bPAC- resulting from high GC-exposure in the parental generation. Supporting this notion, even though bPAC- was not exposed to a high level of GC, some of the DEGs at 6 dpf in bPAC- are involved in processes that potentially regulate GC inactivation (Supplementary Fig. 2b, Supplementary Table 3).

Developmental GC exposure leads to downregulation of transcripts involved in nervous system development

To identify molecular consequences of developmental GC exposure, we characterized DEGs in bPAC+ compared to the wild type. Upregulated DEGs in bPAC+ fish brains at 6 dpf were enriched in the GO terms that include potential regulatory enzymes for GC such as cytochrome monooxygenases, 11β-hydroxysteroid dehydrogenase or sulfotransferases[35-37] (left panel of Fig. 3d). At 13 dpf, the top-ranked enriched biological processes for upregulated DEGs were cell cycle, chromosome segregation, and DNA metabolic process which can cause genomic instability by replication stress[38] (left panel of Fig. 3d). At 120 dpf, bPAC+ brains showed upregulation of genes involved in ribonucleoprotein complex biogenesis, peptide metabolic, translation, protein modification, RNA processing and more, suggesting effects of developmental GC exposure on gene expression regulation and post-transcriptional modification in adulthood (left panel of Fig. 3d, Supplementary Table 3). In contrast, downregulated DEGs across all time points were mainly enriched in processes related to cell adhesion and cell migration, as well as nervous system development including neuron differentiation, cell morphogenesis, and axon development (right panel of Fig. 3d, Supplementary Table 3). Brain-wide analyses using qPCR and immunohistochemistry confirmed alteration of neurogenesis in larval bPAC+ fish. These results are reported in detail in a separate report[39]. During larval stages, downregulated DEGs were also enriched in anatomical structure formation, muscle structure development, vasculature development, external encapsulating structure organization, and more. We also identified functions associated with time-point-specific downregulated DEGs (Supplementary Table 3). For example, DEGs at 6 dpf were enriched in brain development while DEGs at 13 dpf were enriched in the cellular response to organic substance (right panel of Fig. 3d). At 120 dpf, we

identified downregulated DEG enrichments in synaptic signaling, regulation of molecular function, and regulation of GTPase activity and more (right panel of Fig. 3d).

These results highlight long-lasting downregulation of neuronal development and signaling processes, and adult-onset alteration of transcription and translation regulation following developmental GC exposure.

bPAC+ fish show an enhanced endocrine response to acute stress in adulthood

To assess whether high GC exposure during development affects the stress response in adulthood, we subjected wild-type, bPAC+, and bPAC- fish to a looming dot stimulus (LD), which mimics an approaching predator[22] (Figs. 4a,b). Interestingly, bPAC+ adults exhibited a much greater cortisol increase after LD exposure compared to wild-type adults (Fig. 4c), indicative of a sensitized endocrine response to acute stress in bPAC+. Similarly, we found an altered transcriptional response of stress-associated genes in bPAC+ after LD exposure (Fig. 4d). For example, the expression of *nr3c2*, the gene coding for the mineralocorticoid receptor (MR), was further downregulated only in bPAC+ adults following LD exposure, although basal expression of *nr3c2* was already decreased in bPAC+ compared to wild type. In contrast, the GC-inactivating enzyme coding gene, *hsd11b2*, was significantly upregulated in the wild type after LD exposure, but upregulated to a lesser degree in bPAC+ following LD exposure. Together, the significant increase in LD-induced cortisol level and altered expression of *nr3c2*, *fkbp5*, and *hsd11b2* indicate an enhanced endocrine response following acute stress exposure in adult bPAC+.

Developmental GC exposure primes large-scale differential gene expression following acute stress in adulthood

To identify the transcriptional response to acute stress following developmental GC exposure, we carried out whole-brain RNA sequencing of wild type, bPAC+ and bPAC- that had been exposed to LD in adulthood and made three separate comparisons (Figs. 4e,f). First, we compared the wild-type transcriptome at basal (pre-LD) and after LD exposure (post-LD), to identify the normal acute stress response in wild-type fish (referred to as DEG1). Secondly, we compared pre-LD and post-LD in bPAC+ (referred to as DEG2), and finally the post-LD transcriptome of wild type and bPAC+ was also compared (referred to as DEG3). DEG3 includes LD-induced DEGs (overlapping with DEG1 and DEG2) as well as those DEGs that were already altered in bPAC+ prior to LD exposure and therefore the number of DEG3 genes (2034 genes) was greater than that of DEG1 and DEG2 (Fig. 4g). Strikingly, bPAC+

showed more than 20-fold greater number of DEGs in response to LD exposure (DEG2, 1617 genes) compared to wild type (DEG1, 64 genes) (Fig. 4g). Altogether we identified 2515 genes that appeared to be altered in the adult brain by developmental GC exposure in bPAC+, and refer to them collectively as adult bPAC+ DEGs.

The exaggerated transcriptional LD response in bPAC+ indicates that developmental GC exposure in zebrafish primes enhanced alteration of gene expression in adulthood, similar to observations in previous mammalian ELS or GC-primed gene studies[12,13]. To define GC-primed genes, we took the entire DEG2 set and excluded 44 genes that overlapped with DEG1 as these represent normal acute stress-induced genes present also in the wild type. To this gene set, we combined the 18 genes in DEG1 that do not overlap with DEG2 as these genes are reflective of wild-type responses that are missing or altered in bPAC+ (Fig. 4g, Supplementary Table 4). This set of 1591 genes is collectively referred to as GC-primed genes here and represents those genes that are primed by developmental GC exposure to produce an altered transcriptional response to acute stress in adulthood. Among the 1591 GC-primed genes, 1337 downregulated genes were enriched in neuron projection development, synaptic signaling, receptor complex, synapse, metal ion transmembrane transport, and more (Fig. 4h, Supplementary Table 5).

To compare GC-primed genes in zebrafish with those identified in humans, we carried out a comparison with a recent study that reported 702 GC-primed DEGs linked with long-lasting differentially methylated sites following re-exposure to GC (dexamethasone)[13]. These DEGs were identified in GC-treated mature neurons which were exposed to GC during neurogenesis in a human hippocampal progenitor cell (hpc) line. Through the comparison between 702 DEGs (hpc_ELS-primed genes, up:368, down:334)[13] and our DEG3, which represents a similar GC re-exposed condition, we found 163 overlapping genes (up/down=99/64).

Together, these results reveal for the first time a brain-wide set of genes primed by developmental GC exposure. Further, we found a significant overlap between subsets of GC-primed genes identified in zebrafish with those from mammalian studies, suggesting a high degree of evolutionary conservation of molecular mechanisms underlying reprogramming by GC.

Adult bPAC+ DEGs are associated with neuropsychiatric disorders in humans

Since stress exposure during development is a risk factor for the development of psychiatric disorders in humans[6-8], GC-primed genes were compared with known psychiatric disease risk

factors using the curated DisGeNET database[40]. Strikingly, human homologs of GC-primed genes exhibit a significant enrichment of known psychiatric disease-associated genes (Supplementary Table 8). Top-ranked psychiatric disorders were schizophrenia, bipolar disorder, and autistic disorder (Supplementary Fig. 4). Further, the entire adult bPAC+ DEG set was strongly enriched in known psychiatric disease risk factors and in this case depressive disorder was also included in the top four ranked diseases (Figs. 5a,b, Supplementary Table 9). Upon LD exposure, the difference in expression of schizophrenia-associated genes in bPAC+ was greatly enhanced, as represented by the size of nodes in Fig. 5c. Further, schizophrenia-associated adult bPAC+ DEGs were enriched in either of two KEGG pathways: neuroactive ligand-receptor interaction (FDR=2.01e-08), or MAPK signaling pathway (FDR=9.74e-08). Overall, across all four disease categories, the top-ranked disease-associated adult bPAC+ DEGs were mainly enriched in the downregulation of synaptic signaling, and multicellular organismal process (Fig. 5d).

Adult bPAC+ DEGs associated with schizophrenia, bipolar disorder, depressive disorders, and autistic disorders were predominantly downregulated following LD exposure (Fig. 5e). Interestingly, we noted that some of the disease-associated adult bPAC+ DEGs were already differentially expressed from the larval stage (trans-temporal, 60 genes) while some others showed alteration in their expression only upon exposure to acute stress in adulthood (adult-onset, 65 genes) (Fig. 5f). Other non-disease-associated adult bPAC+ DEGs maintained differential expression from the larval stage (540 genes) or exhibited an enhanced alteration upon adulthood stress (adult-onset, 463 genes) (Supplementary Table 10). Trans-temporal adult bPAC+ DEGs were mostly enriched in axon development (FDR=1.33e-08, 31 DEGs), whilst adult-onset adult bPAC+ DEGs were enriched in ion transport (FDR=7.26eE-05, 44 DEGs) (Supplementary Table 11). Interestingly, a cell-type enrichment test using the human homologs of adult bPAC+ DEGs showed that 24 trans-temporal adult bPAC+ DEGs were associated with non-microglial oligodendrocyte lineages. In contrast, the downregulated adult-onset adult bPAC+ DEG set was associated with non-microglial neuron-enriched genes including GABAergic receptor-associated genes (*GABRA1*, *GABRG2*), other ion channel activity genes (*SLC12A5*, *CACNA1E*, *CACNA1I*, *UNC80*, *KCNH1*, *SCN2B*), and genes associated with synapse and neuronal cell signaling (*DNM1*, *DLGAP2*, *RPH3A*, *GPR158*) (Supplementary Table 12). More than half of those genes were GC-primed genes. These results indicate that oligodendrocyte lineage cells are consistently suppressed following developmental GC-exposure, and developmental GC primes suppression of neuronal signaling primarily in adulthood when encountering stressful stimuli.

Altered gene expression associated with epigenetic modifications and RNA processing in bPAC+ fish

To identify underlying mechanisms by which developmental GC exposure regulates gene expression in the brain, we analyzed the expression of epigenetic modifiers. We identified 70 adult bPAC+ DEGs associated with epigenetic modifications (Supplementary Figs. 5 a,b, Supplementary Table 13). These genes are associated with RNA modification (up/down=3/3), RNA processing including mRNA splicing (up/down=27/17), histone modification (up/down=4/18), and DNA modification (up/down=0/3). Among them, the DNA methyltransferase (DNMT) 3aa gene and the histone modification-associated genes, *chd3*, *hdac4*, *hdac5*, *kdm2ba*, *kdm4aa*, *mta3*, *nsd3*, *padi2*, *suv39h1b*, and *yeats2*, showed consistent downregulation across time-points.

We also found significant differences in the expression of 4 out of 7 *DNMTs* in the zebrafish genome including *dnmt1*, *dnmt3aa*, *dnmt3ab*, and *dnmt3bb.3* across time points and conditions in bPAC+ compared to the wild type (Supplementary Fig. 5c). In the wild type, *dnmt1* is highly expressed at 6 dpf and expression then gradually decreases over time, but in bPAC+ expression was significantly reduced at 6 dpf. Expression patterns of *dnmt3aa* and *dnmt3ab*, orthologous to human *DNMT3A*, known as one of the writers of *de novo* methylation, mainly showed differences in bPAC+ adult brains. Further, low expression of *dnmt3aa* and upregulation of *dnmt3ab* was observed upon LD exposure in adulthood in bPAC+. To determine whether alteration of the most differentially regulated epigenetic modulators (labeled with * in Supplementary Fig. 5a) lasts throughout adulthood, we analyzed their expression at 8 mpf, with and without LD exposure. Downregulated expression of those genes was continuously observed in the later adult stage (8 mpf) mainly upon acute stress in bPAC+ adults (Supplementary Fig. 5d). These results suggest that developmental GC exposure leads to pervasive, long-lasting changes to epigenetic modulators.

Social behavior-associated genes are a part of the GC-primed genes

To search for a potential link between the observed molecular changes and social behavior, we analyzed the expression patterns of 101 known social behavior-associated genes from the Gene Ontology Annotation database (henceforth referred to as social genes). Interestingly, 54 of these social genes were differentially expressed in bPAC+ brains, where 44 were downregulated at least at a single time point ($|FC|>1.5$ and $FDR<0.05$) and only 11 were

upregulated (*npas4a* was double counted because downregulated at 6 dpf and upregulated at 13 dpf, Fig. 6a, upper panel). In the basal condition, 6 dpf brains showed the largest change (up/down=2/31). The most significant downregulated social genes over time were *nlgn2b*, *mapk8ip2*, *brinp1*, *slc6a4a*, as well as oxytocin (*oxt*), a well-known social gene[41] (Fig. 6a). bPAC- showed a similar trend in the downregulated gene expression pattern following LD exposure as in bPAC+, albeit the level of difference was lower though. (Fig. 6a, bottom panel). We confirmed the downregulation of *oxt* both in larval and adult bPAC+ brains using qPCR (Fig. 6b). Downregulated *oxt* expression and the number of oxt+ cells in larval brains were also confirmed using immunohistochemistry which showed preferential reduction of the posterior cluster of oxt+ cells which is likely to be similar to mammalian parvocellular oxt+ neurons based on their projection[42, 43]. (Figs. 6c-e).

Using differentially expressed social genes at 6 dpf, we performed network-based analyses using the String database[44] (Fig. 6f). The identified protein network was enriched in modulation of chemical synaptic transmission, synaptic signaling, learning and memory, pre-synapse assembly, and social behavior. Notably, *oxt* regulators including *myt1la*, *med12*, and *thrab* were downregulated at 6 dpf and two of them (*myt1la*, *med12*) remained downregulated until adulthood (Fig. 6g). Further, transcription factor (TF) enrichment analysis of human orthologues of the social genes identified *myt1la* as one of the top regulators of 47 differentially expressed social genes, based on the ChEA3 database[45] (Fig. 6h). These results suggest that downregulation of *myt1la* in bPAC+ may result in the altered expression of social genes including *oxt*[46].

Strikingly, a subset of social genes was also part of the GC-primed genes indicating that the regulation of their adult expression is highly sensitive to the history of developmental GC exposure (red label in Fig. 6a). Next, we performed a TF enrichment test of GC-primed genes using the ChEA3 platform[45] and identified common potential upstream regulators of GC-primed genes (Figs. 6i,j, Supplementary Table 6). For the upregulated GC-primed genes, immediate early genes were top-ranked TFs. Among the top-ranked TFs for downregulated GC-primed genes, *camta2* and *myt1l* may be involved in the regulatory process of 149 and 139 downregulated genes, respectively. Expression of *myt1l* was consistently downregulated in bPAC+ adults at both pre-LD and post-LD condition (Fig. 6k). Together, these results suggest developmental GC-induced regulation of *myt1la* as one potential mechanism by which stress in early life alters adult social behavior and primes expression of brain-wide gene networks.

Discussion

This study examines the effects of chronic developmental GC exposure in shaping adult phenotypes at both behavioral and molecular levels. We report altered goal-directed behaviors in adults and molecular consequences of GC-exposure in both larvae and adults. Further, we report a comprehensive identification of genes differentially expressed in adulthood following developmental GC exposure, many of which correspond to genes associated with neuropsychiatric disorders in humans. These findings suggest that developmental GC exposure primes differences in GR (acute stress)-induced transcriptional activation in adulthood and may increase the risk for neuropsychiatric disorders by affecting a network of stress-sensitive genes.

Our bPAC+ model involves the optogenetic activation of *star*-expressing interrenal cells, leading to an increase in endogenous cortisol levels in transgenic animals. Previous studies in fish using LC-MS have not detected MCs in any significant quantities in either larval nor adult samples[16-19], suggesting that the zebrafish interrenal produces no/negligible amounts of MC. Similarly, androgens are not detectable in zebrafish larvae[16], but are detectable in adult male whole-body samples [17-19], suggesting that androgens are only produced in the testes in zebrafish. As such, the phenotypes observed in our bPAC+ model are primarily due to the effects of GC and its downstream effectors, rather than due to MC or androgen over-exposure. The increase in cortisol in our model is observed from 2 dpf onwards, consistent with the onset of *star* gene expression. 2 dpf is considered to represent an early postembryonic development stage comparable to fetal and neonatal stages in mammals[47, 48]. As zebrafish develop externally, it is not possible to distinguish pre- vs. post-natal stress exposure. Elevated cortisol level is maintained until 2 months of development and decreases with the onset of adulthood. This may be due to the decrease in blue-light penetration following the reduced transparency of the skin as fish grows.

We report enhanced endocrine sensitivity of adult bPAC+ fish in response to acute stress. Further, we observed a significant decrease in the expression of *nr3c2,* which encodes the high GC-affinity MC receptor (MR), both at pre- and post-LD exposure. Given the higher affinity of MR to GC, MR can be a regulator of GR signaling in MR and GR co-expressing cells in basal conditions[49,50]. Moreover, reduced transcriptional reactivity of *hsd11b2,* a key enzyme for cortisol inactivation[51,52], in bPAC+ after LD may lead to an increased accumulation of active cellular cortisol. These results suggest that developmental GC-

exposure may lead to a sensitized response to stress by decreasing MR expression at pre- and post-LD and attenuating the increase of *hsd11b2* expression.

We identified brain-wide alterations in bPAC+ through whole-brain transcriptome analyses using both larval and adult zebrafish. Downregulated genes in bPAC+ are mainly enriched in cell morphogenesis, neuronal cellular signaling, and neuropsychiatric disorders-associated gene sets. Despite the differences in species, stressor type, and brain region, the results of our GO enrichment test and observed phenotypes are broadly similar to previous findings obtained from ELS rodent models which report alterations in axon development, synaptic density and plasticity, altered hippocampal mossy fibres[4, 5, 53]. However, we identified a greater number of DEGs when compared to previously reported mammalian ELS paradigms consistent with the fact that the model described here represents a stronger and longer stress exposure.

Interestingly, we identified a large set of adult-onset upregulated DEGs which were enriched in DNA metabolism, various RNA processing processes, and posttranslational modifications. This may indicate that developmental GC-exposure can lead to genome instability by DNA replication stress and altered gene expression regulation via alternative splicing, non-coding RNAs, and posttranslational modifications in adulthood. Moreover, in adult bPAC+, we identified oligodendrocyte lineage cell markers in downregulated trans-temporal DEGs while neuron-enriched genes were enriched in downregulated adult-onset DEGs. This result suggests that developmental GC-exposure has a cell-type-specific impact.

Identified adult bPAC+ DEGs were enriched in neuropsychiatric disorders when compared to a publicly available catalogue of disease-associated genes based on gene expression network, eQTLs and GWAS[40]. The majority of schizophrenia, bipolar disorder, depressive disorder, and autistic disorder-associated adult bPAC+ DEGs showed an exaggerated transcriptional response to stress in adult bPAC+ brains. Those DEGs were overrepresented in synaptic signaling processes including genes for GABA receptors (*gabrg2*, *gabbr1a*, *gabbr1b*, *gabbr2*, *gabra1*, *gabra2a*, *gabra4*, *gabra5*, *gabrg1*, *gabrg2*), glutamate receptors and transporter (*grm1b*, *grm3*, grm5b, *slc17a7a*), dopamine receptors and transporter (*drd1b*, *drd2a*, *drd2b*, *slc6a3*), serotonin receptor (*htr1b*), cholinergic receptor (*chrm2a*), opioid receptor (*pnoca*), and neurexins (*nlgn1*, *nlgn2b*, *nlgn4xb*). In a human study, genetic variants associated with GR-mediated immediate transcript response were able to predict risk for psychiatric disorders

including depression[14]. Consistent with this, our findings suggest developmental GC exposure leads to differences in GR-induced transcriptional activation in adult brains and may mediate the risk for psychiatric disorders by altering a network of neuronal signaling-related stress-sensitive genes.

Growing evidence indicates that GC signaling can lead to modifications to DNA methylation, histone codes, and non-coding RNAs[54-56]. Similarly, we identified significantly altered expression of epigenetic modulators. One of the most significantly altered epigenetic regulators in bPAC+ brains are *DNMTs* (*dnmt1* and *dnmt3aa*) at the larval stage (6 dpf), which may result in a global or local reduction in DNA methylation. DNMT1 mainly plays a role in the maintenance of DNA methylation[57] while accumulation or gain of DNA methylation is achieved by DNMT3a and -3b[58]. Given such far-reaching effects of each DNMT, alteration of expression levels of dnmt genes that we observe in bPAC+ is predicted to change the global DNA methylation landscape across the life course. In our study, we identified consistently downregulated adult bPAC+ DEGs associated with histone modifications across the life course. Temporal regulation of chromatin structure via the "histone code" in neurons has been regarded as a crucial factor for dynamic plasticity in the mature nervous system and long-lasting neuronal responses[59]. Among the histone modulator genes, *hdac4* and *hdac5* were consistently downregulated in bPAC+ fish, and have been implicated in depression, fear learning, PTSD, eating disorder (Anorexia nervosa), and schizophrenia[60-65]. Furthermore, many epigenetic-associated adult bPAC+ DEGs are involved in RNA processing and RNA editing. Among them, we found a downregulation of *adarb2* and *pum2* in bPAC+ fish after exposure to LD. Chen et al. suggest that ADARB2 has a regulatory role in RNA editing by inhibiting the binding of other adenosine deaminases acting on RNA (ADARs)[66] and ADARs are associated with the development of epileptic seizures in mice and depressive phenotypes[67,68]. Further, *pum2* is involved in axon growth and branching[69]. However, further investigation is required to reveal direct mechanisms between the identified adult bPAC+ DEGs and alterations of epigenetic modulators.

As early exposure to excess GC is considered a global risk factor for the development of mental disorders, our behavioral pipeline was tailored to assess performance in key behavioral manifestations rather than disease-specific tasks. bPAC+ fish did not show defects in aspects of perception as they can recognize visual and olfactory stimuli in the form of social cues and food pellets. However, bPAC+ fish showed deficits in fear learning using a

pavlovian conditioning paradigm that pairs electric shock with the color green. It is possible that perceived salience of electric shock as a stressor rather than fear learning is diminished in bPAC+. However, we consider this possibility less likely as bPAC+ can respond robustly to acute stress presented in the form of LD. This phenotype is consistent with what has been reported in rodent ELS models, which showed deficits in fear learning, learning impairment, and decreased cognitive flexibility[70-72]. In zebrafish, fear-conditioned avoidance learning is a robust phenotype whereby fish exhibit freezing and erratic behavior upon re-exposure to the conditioned stimulus (CS)[73, 74]. Although one study found that chronic unpredictable stress did not have any effect on fear conditioning in adult zebrafish[75], a different ELS protocol found improvement of short-term memory by early life stress when testing in the unconditioned y-maze[76]. Differences in how zebrafish learn fear association are dependent on whether they are reactive or proactive responders to stress, raising the question of how bPAC+ fish's response fits into that spectrum[77].

To assess how bPAC+ responds in a novel environment, we utilized the novel tank test which has been compared to the open field test in rodents. A well-reported typical response of wild-type fish in the novel tank assay consists of immediate dive to the bottom, sustained depth-preference, and erratic bouts of fast swimming[78,79]. Both depth-preference and erratic bouts are enhanced with increased levels of stress and can be ameliorated by treatment with anxiolytic compounds[80-82]. bPAC+ fish showed less depth preference throughout the trial and fewer erratic swimming bouts compared to wild types, suggesting altered novelty perception and processing in bPAC+ fish. This outcome further supports the findings in the fear conditioning assay, that bPAC+ fish may possess dysfunctional fear processing and/or novelty adaption mechanisms.

bPAC+ fish exhibited a reduction in the maintenance of social interactions in the absence of social recognition or approach deficits using our new social approach and maintenance assay[22]. Both a reduction in the time spent in the interaction zone and the number of interactions performed by bPAC+ fish were observed. Similarly in previous zebrafish ELS studies, diminished shoaling behavior was observed following early-life social isolation and was associated with both motivational deficiencies linked to dopaminergic systems[83] and increased sensitivity to stimuli which was reversed by anxiolytic treatment[84]. Impairments in social behavior or social withdrawal are common among different psychiatric disorders,

including autism[85] and schizophrenia[86], and have been described in rodent models of ELS in which deficits in social interaction in the absence of social recognition and approach have been observed[87]. Interestingly, neonatal handling and short bouts of maternal separation in male rats resulted in reduced oxytocin neurons in the parvocellular region of the paraventricular nucleus (PVN) of the hypothalamus and a reduction in the time spent interacting and sniffing, with no differences in recognition or approach reported[87]. We observed reduced expression of oxytocin, in bPAC+ brains during the larval stage, and this downregulation persisted until adulthood, although the extent of the difference decreased over time. Consistent with our observation that posterior oxt+ cluster is selectively reduced in bPAC+, a subset of parvocellular oxt neurons enriched genes identified in recent scRNA-seq results of mice were downregulated in bPAC+ (such as *brinp1*, *baiap3*, *pacsin1a*, *syt17*, and *rcan2*)[88]. This may suggest that subgroups of oxt+ neurons are differentially controlled by ELS during development and play distinct roles in ELS-induced modification of stress responses and social behavior.

In conclusion, we show that developmental GC exposure leads to altered behaviors and brain-wide transcriptional changes that were enriched in the processes of DNA metabolism, nervous system development and neuronal signaling across the life course. We identified brain-wide adult bPAC+ DEGs that are overrepresented in gene sets associated with neuropsychiatric disorders and are enriched in epigenetic modulators. Further, developmental GC exposure led to exaggerated endocrine responses and primed a set of genes to exhibit altered transcriptomic responses to subsequent challenges in adult fish. Lastly, we identified reduced expression of *oxt* and *mytl1a* in bPAC+ which may contribute to the altered social behavior phenotype in bPAC+. We propose that the brain-wide DEG set identified here will be useful for guiding future studies dissecting the mechanism underlying developmental GC-exposure and behavioral dysfunction and neuropsychiatric disease susceptibility in adulthood. The zebrafish model presented here provides an opportunity to dissect the long-term effects of endogenous GC elevation during development and can be leveraged in future studies to identify critical period, intensity, and duration of developmental GC exposure that leads to adult dysfunction.

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Author contributions

SR conceived the project. MC and SR designed the project. MC acquired and analyzed transcriptomic data. AC acquired and analyzed behavior data. SK, HE, AT and SK contributed to reagent generation or data acquisition. MC, SR, and AC drafted the article. MC, HE, and SR critically revised the manuscript. All authors approved the submission.

Competing interests

SR holds a patent, European patent number 2928288 and US patent number 10,080,355: “A novel inducible model of stress.”. The remaining authors declare no known competing interests.

Figure legends

Figure 1. Ambient light-exposed bPAC+ fish show elevation of cortisol during development. (a) An image of *Tg(star:bPAC-2A-tdTomato)*$^{+/-}$ larva at 5 dpf. The transgene is expressed solely in the interrenal gland (arrow). The schematic represents the intracellular events in the steroidogenic interrenal cells of transgenic fish (dotted box). Endogenous GC production is stimulated by ACTH signaling. Similary, bPAC mimics ACTH signaling by increasing cAMP when exposed to blue light. (b) Fish were raised under ambient aquarium lighting conditions with white fluorescent light (12-hour light and 12-hour dark). Elevation of basal GC level was observed at larval and juvenile stages. (c) White ambient light (blue+) elevates the basal GC level of 6 dpf bPAC+ larvae. ***=P<0.001, two-tailed unpaired t-test. However, there was no significant difference in GC level when fish were raised under filter restricting blue light spectrum within the ambient light (blue-). (d) One of the well-known GR signaling markers, *fkbp5*, was significantly upregulated in bPAC+ larvae (P = 0.016) compared to wild-type control, two-tailed unpaired t-test. * = P<0.05. Basal GC level at larval stages (e) and from juvenile to adult (f). Abbreviations: GC, glucocorticoid; ACTH, Adrenocorticotropic Hormone; AC, adenylyl cyclase; cAMP, Cyclic adenosine monophosphate

Figure 2. Reduced social interaction in adult bPAC+ fish. No significant differences were observed in average speed (a) nor turn frequency (b) among wild type, bPAC+ and bPAC-. (c) Schematic figures for the social approach and maintenance test. During the pre-social phase, no significant difference in color preference (d) nor depth (e) was observed. (f) Representative traces of wild-type fish during the social phase. The green shade indicates the interaction zone. (g) During the social phase, no significant difference in approach rate to conspecific fish between groups. Log-rank test (Mantel-Cox, P = 0.77). (h) Representative traces of wild-type and bPAC+ fish during the social phase. bPAC+ and bPAC- showed a significantly reduced duration (i) and frequency of social interactions (j) compared to the wild type. Dunnett's multiple comparisons followed one-way ANOVA, a) F(2, 27)=0.2248, P=0.80; b) F(2, 27)=2.332, P=0.12; d) F(2, 98)=0.6224, P=0.54; e) F(2, 98)=1.801, P=0.1706; i) F(2, 98)=6.273, P=0.0027; j) F(2, 98)=10.93, P<0.0001.

Figure 3. Developmental GC exposure leads to differential gene expressions across the life course (a) Venn Diagram for up- and down-regulated DEGs between wild type and bPAC+; criteria for DEG identification: |FC|>2 and FDR <0.01. A lower FC threshold, |FC|>1.5, was applied for samples at 120 dpf. (b) Average read counts of genes by genotype and time points are represented by z-score in the heat map. Bars on the right side indicate the paternity of fish. (c) Expression profiles of GC-associated genes. *=P<0.05, **=P<0.01, ***=P<0.001, ****=P<0.0001. Tukey's multiple comparisons test followed two-way repeated measures ANOVA. (d) Top enriched biological processes for up- and down-regulated DEGs in bPAC+. d6: 6 dpf, d13: 13 dpf, d120: 120 dpf, wt: wild type, FC: fold change, GC: glucocorticoid, *adj.p*: adjusted P-value, ratio: identified DEGs/the number of genes included in the term. Complete DEG-enriched GO terms are described in Supplementary Table 3.

Figure 4. Developmental GC exposure primes differential gene expression following acute stress in bPAC+ adults. (a) Schematic of the acute stress paradigm. Looming dots (LD) mimic approaching predators from above (Cook et al. 2023). (b) Schematic for acute-stressed and control sample collection (arrows). Acute stress is induced by looming dot (LD)-exposure in adults (120 dpf). post-LD samples were collected after 15 min of LD exposure.

Yellow striped box: bPAC+ activation; yellow stars: LD. (c) bPAC+ showed significantly higher GC levels in post-LD conditions, compared with basal. Tukey's multiple comparisons followed one-way ANOVA; F(5, 54)=19.31, $P<0.0001$. (d) Expression profiles of GC-induced genes following LD exposure. *= $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$ from multiple two-tailed unpaired t-tests. Error bars indicate mean+SEM. (e) Schematic for three comparisons to identify transcriptional changes in response to LD exposure in adults. DEGs were determined by FC and FDR. |FC|>1.5 and FDR<0.01 for DEG1 and 2; |FC|>2 and FDR<0.01 for DEG3. (f) z-score for mean CPM of adult bPAC+ DEGs is visualized in a heatmap. (g) Venn diagram for adult bPAC+ DEGs. The list of DEGs is described in Supplementary Table 4. The adult bPAC+ DEGs = DEG1 + DEG2 + DEG3 - (DEG1 ∩ DEG2). GC-primed genes = DEG1 + DEG2 - (DEG1 ∩ DEG2). The red asterisk represents the overlap (2 genes) between DEG1 and DEG3. (h) The top 3 GO terms enriched in downregulated GC-primed genes. The enrichment score = $-\log_{10}$(adjusted *P*-value). The complete results of the functional enrichment test are described in Supplementary Table 5.

Figure 5. Adult bPAC+ DEGs are enriched in gene sets associated with human neuropsychiatric disorders. (a) Result of human disorders enrichment test based on the DisGeNET database. The ratio represents overlapping/input genes. Top ranked disorders are schizophrenia (FDR= 1.61e-04, ratio (adult bPAC+ DEGs/disease genes)=170/883), bipolar disorder (FDR= 7.48e-04, ratio=100/477), depressive disorder (FDR= 2.89e-03, ratio=63/289), mental depression (FDR= 2.87e-03, ratio=59/254) and autistic disorder (FDR= 1.61e-04, ratio=65/261). (b) Venn diagram for adult bPAC+ DEGs that are associated with the top five human psychiatric disorders in panel a. Depressive disorder and Mental Depression-associated genes (as shown in a), are combined and referred to as depressive disorders in b and d. (c) STRING protein-protein interaction (PPI) networks of schizophrenia-associated adult bPAC+ DEGs genes in pre- and post-LD conditions indicate LD-sensitive gene expression. The size and color of the node represent comparative fold change, and gene expression; up (orange) or downregulation (blue), respectively. DEGs enriched in KEGG pathways Neuroactive ligand-receptor interaction (FDR = 2.01e-08) are labeled in yellow whilst those enriched in MAPK signaling pathway (FDR=9.74e-08) are labeled in green. (d) Top GO terms enriched in the top-ranked disease-associated adult bPAC+ DEGs. Adult bPAC+ DEGs that are associated with schizophrenia, depression, autism, and bipolar disorder are mainly enriched in the downregulated functions in synaptic signaling, and multicellular organismal process. The enrichment score = -log10(adjusted P-value). (e) The heatmap depicts the $\log_2$FC of GC-altered genes associated with the top five human psychiatric disorders in panel a (Depressive disorder and mental depression are merged). The numbers in the brackets represent the number of adult bPAC+ DEGs. 6, 13, and 120 denote dpf, and LD denotes post-LD at 120 dpf. (f) Subsets of adult bPAC+ DEGs associated with the top five diseases in a show consistent differential expression across the life course (significant at all time points, labeled trans-temporal) or only in adulthood (significant only in adulthood, labeled adult-onset). Genes that exhibited expression patterns outside of these conditions were excluded.

Figure 6. Social behavior-associated genes tend to be downregulated in bPAC+ fish across the life course. (a) The expression of social behavior-associated genes, log2FC from two comparisons, bPAC+ vs. wild type, and bPAC- vs. wild type are represented by a heatmap. Asterix in each cell denotes that FDR < 0.05. Red labels indicate GC-primed genes. (b) Decreased *oxt* expression in bPAC+ was confirmed both in larval and adult brains by real-time qPCR. * = $P<0.05$, multiple unpaired t-tests. Error bar indicates mean±SEM. (c) Expression of *oxt* was decreased especially in the medial posterior cluster of oxt+ cells

(yellow arrows) in the neurosecretory preoptic area (NPO) of 5 dpf bPAC+ larvae. a: anterior, p: posterior. scale bar represents 25µm. (d) the signal intensity of *oxt* in NPO was significantly reduced in bPAC+ NPO. (e) the number of oxt+ cells in bPAC+ larvae were significantly less than those of wild type. Mann-Whitney test, d) n=8; e) n=70. (f) Protein-protein interaction network of significant DEGs that are implicated in social behavior at 6 dpf. The color and size of nodes represent log2FC. This protein network was enriched in modulation of chemical synaptic transmission (strength=1.38, FDR=5.44e-12), synaptic signaling (strength=1.33, FDR=1.12e-11), learning and memory (strength=1.67, FDR=1.60e-12), pre-synapse assembly (strength=2.41, FDR=5.44e-11) and social behavior (strength=2.44, FDR=1.17e-30). (g) Log2FC of known oxt regulatory elements. *= FDR < 0.05. (h) Top enriched transcription factors for differentially expressed social behavior-associated genes. Light green represents TFs that are significantly differentially expressed in bPAC+. (i-j) Top enriched transcription factors (TFs) for upregulated (i) or downregulated (j) GC-primed genes are presented as a bar plot. The light green color indicates TFs that are significantly differentially expressed in bPAC+. (k) Expression of *myt1la* is significantly downregulated in in bPAC+ both in pre- and post-LD. ns= $P>0.05$, *=$P<0.05$, **=$P<0.01$, ****=$P<0.0001$, Tukey's multiple comparisons test followed by one-way ANOVA.

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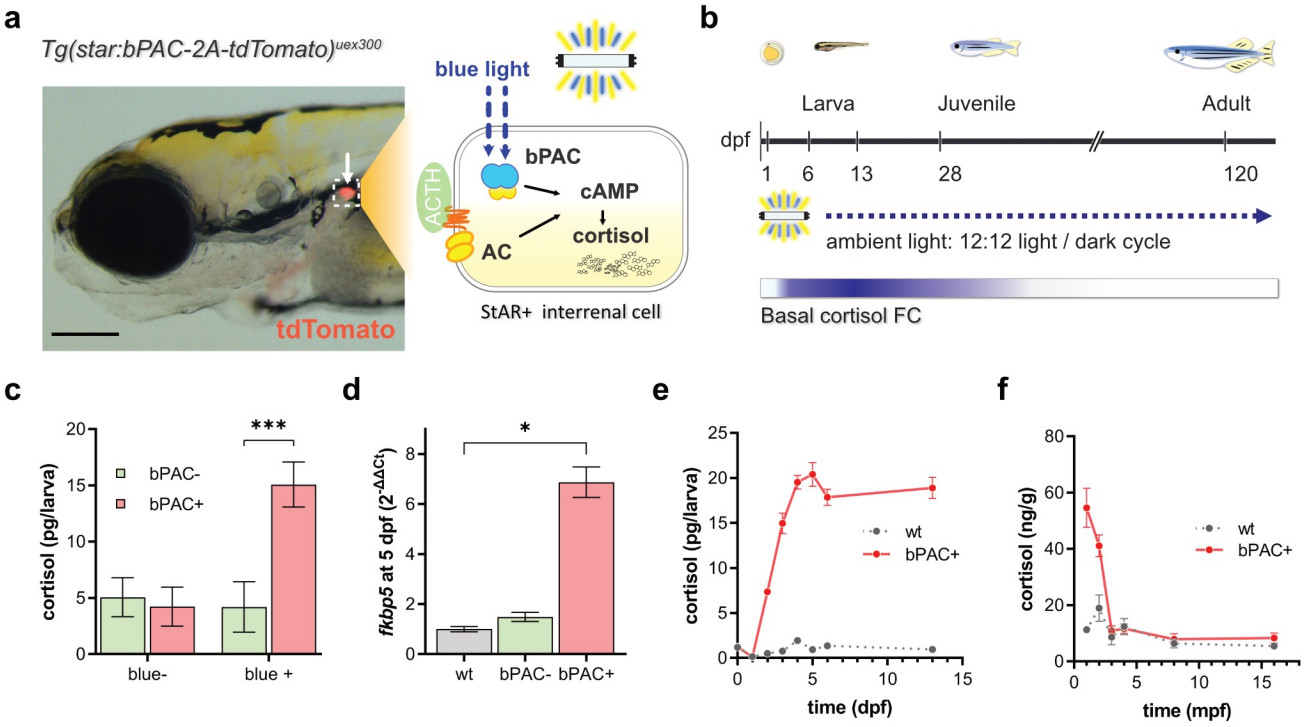

a
Tg(star:bPAC-2A-tdTomato)uex300
tdTomato
blue light
bPAC
cAMP
cortisol
AC
ACTH
StAR+ interrenal cell
b
Larva
Juvenile
Adult
dpf
1
6
13
28
120
ambient light: 12:12 light / dark cycle
Basal cortisol FC
c
cortisol (pg/larva)
bPAC-
bPAC+
***
blue-
blue +
d
fkbp5 at 5 dpf (2-ΔΔCt)
*
wt
bPAC-
bPAC+
e
cortisol (pg/larva)
wt
bPAC+
time (dpf)
f
cortisol (ng/g)
wt
bPAC+
time (mpf)

Figure 1

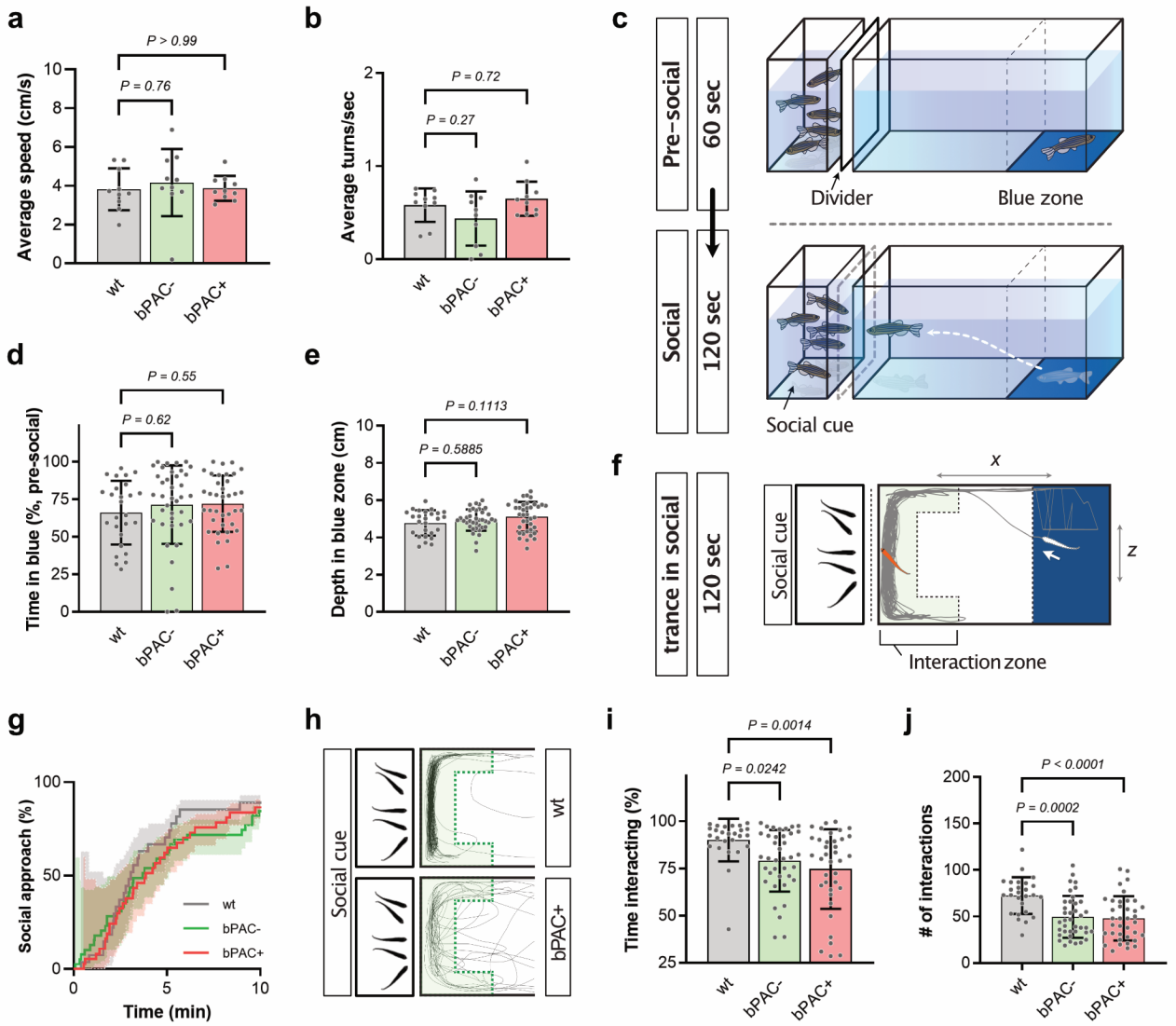

a
P > 0.99
P = 0.76
Average speed (cm/s)
wt
bPAC-
bPAC+
b
P = 0.72
P = 0.27
Average turns/sec
c
Pre-social
60 sec
Divider
Blue zone
Social
120 sec
Social cue
d
P = 0.55
P = 0.62
Time in blue (%, pre-social)
e
P = 0.1113
P = 0.5885
Depth in blue zone (cm)
f
trance in social
120 sec
Social cue
x
z
Interaction zone
g
Social approach (%)
Time (min)
wt
bPAC-
bPAC+
h
Social cue
wt
bPAC+
i
P = 0.0014
P = 0.0242
Time interacting (%)
j
P < 0.0001
P = 0.0002
# of interactions

Figure 2

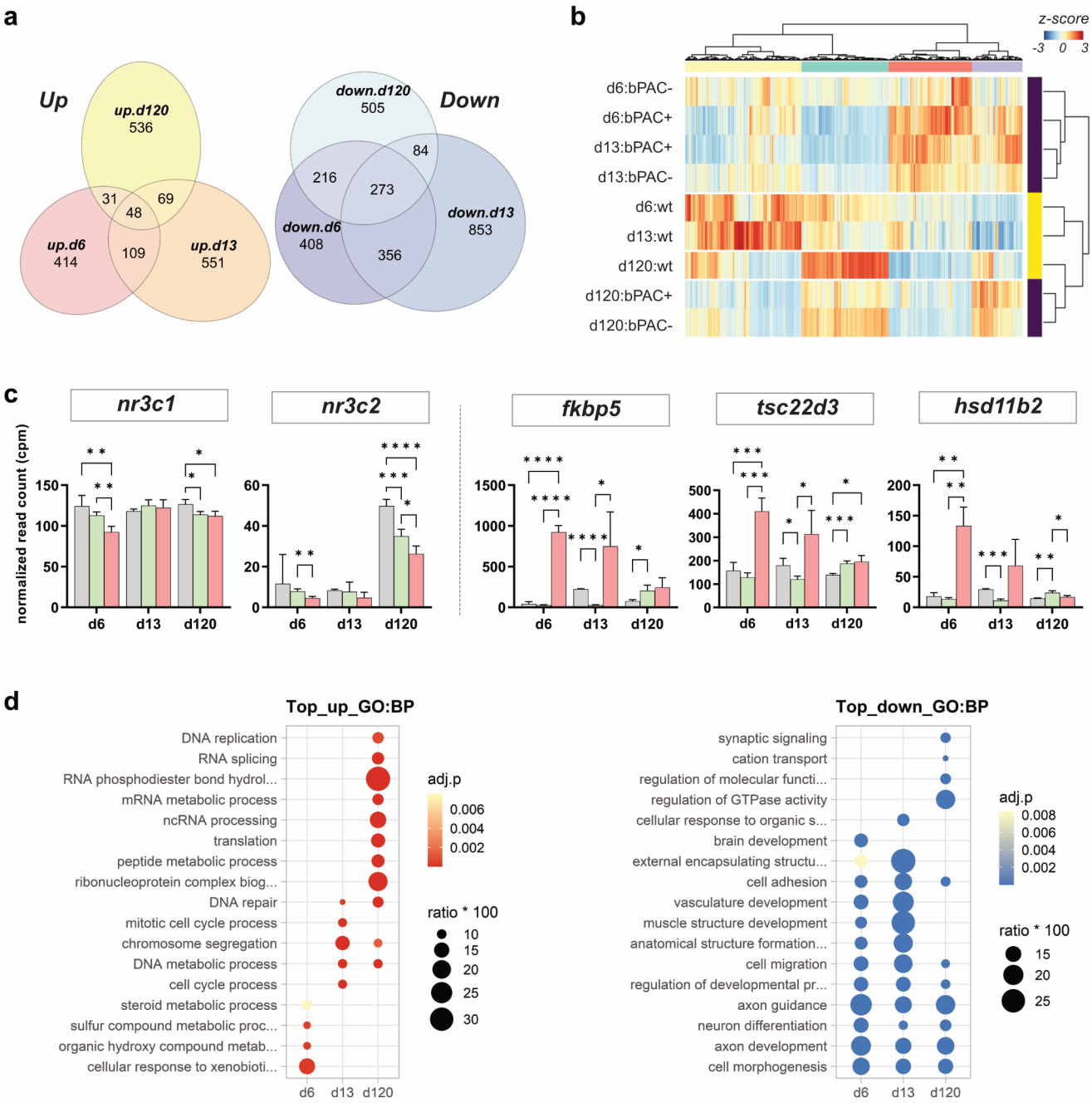

a
Up
up.d120
536
up.d6
414
up.d13
551
31
69
48
109
Down
down.d120
505
down.d6
408
down.d13
853
84
216
273
356
b
z-score
-3 0 3
d6:bPAC-
d6:bPAC+
d13:bPAC+
d13:bPAC-
d6:wt
d13:wt
d120:wt
d120:bPAC+
d120:bPAC-
c
nr3c1
nr3c2
fkbp5
tsc22d3
hsd11b2
normalized read count (cpm)
d6
d13
d120
d
Top_up_GO:BP
DNA replication
RNA splicing
RNA phosphodiester bond hydrol...
mRNA metabolic process
ncRNA processing
translation
peptide metabolic process
ribonucleoprotein complex biog...
DNA repair
mitotic cell cycle process
chromosome segregation
DNA metabolic process
cell cycle process
steroid metabolic process
sulfur compound metabolic proc...
organic hydroxy compound metab...
cellular response to xenobioti...
adj.p
0.006
0.004
0.002
ratio * 100
10
15
20
25
30
Top_down_GO:BP
synaptic signaling
cation transport
regulation of molecular functi...
regulation of GTPase activity
cellular response to organic s...
brain development
external encapsulating structu...
cell adhesion
vasculature development
muscle structure development
anatomical structure formation...
cell migration
regulation of developmental pr...
axon guidance
neuron differentiation
axon development
cell morphogenesis
adj.p
0.008
0.006
0.004
0.002
ratio * 100
15
20
25

Figure 3

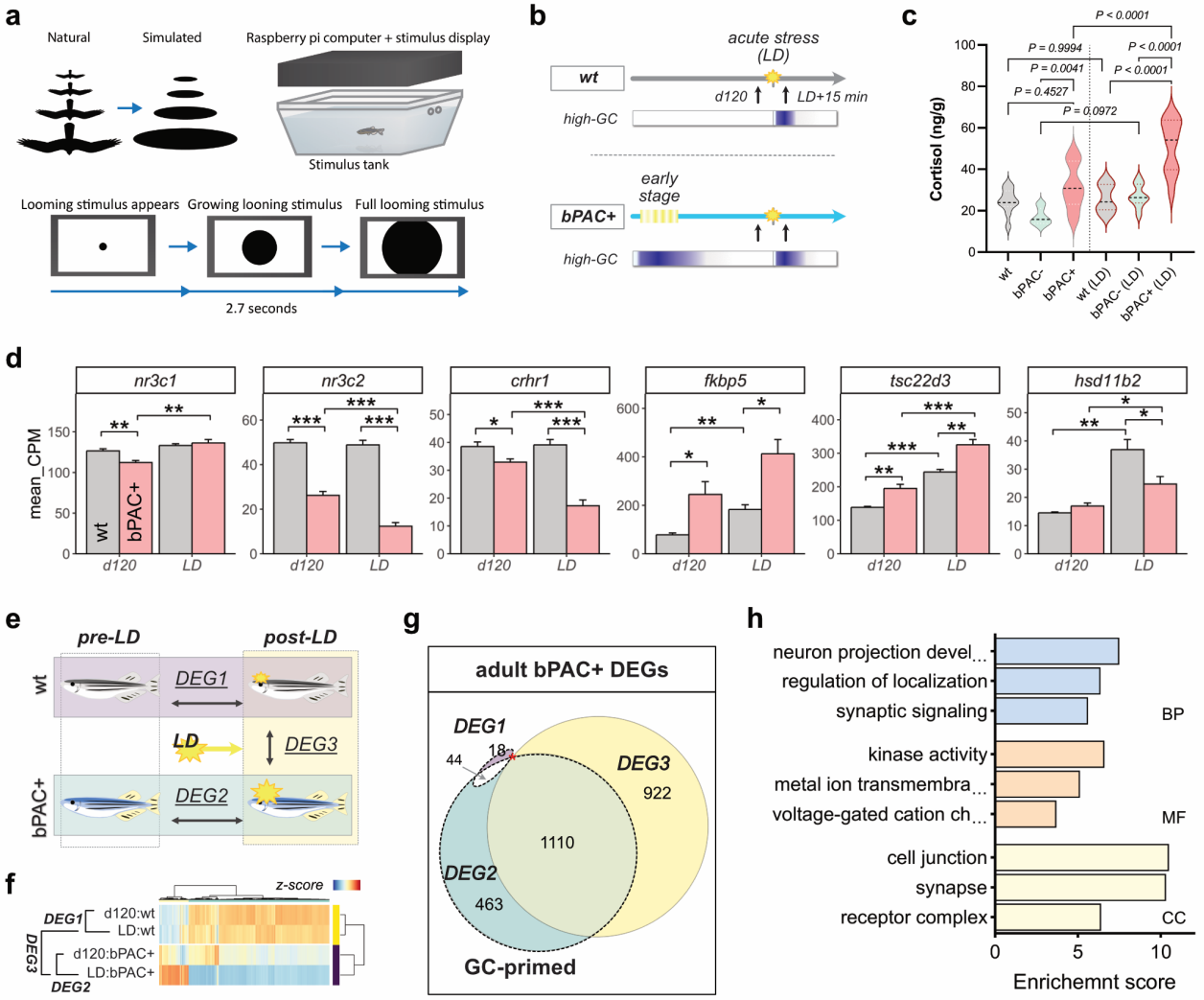
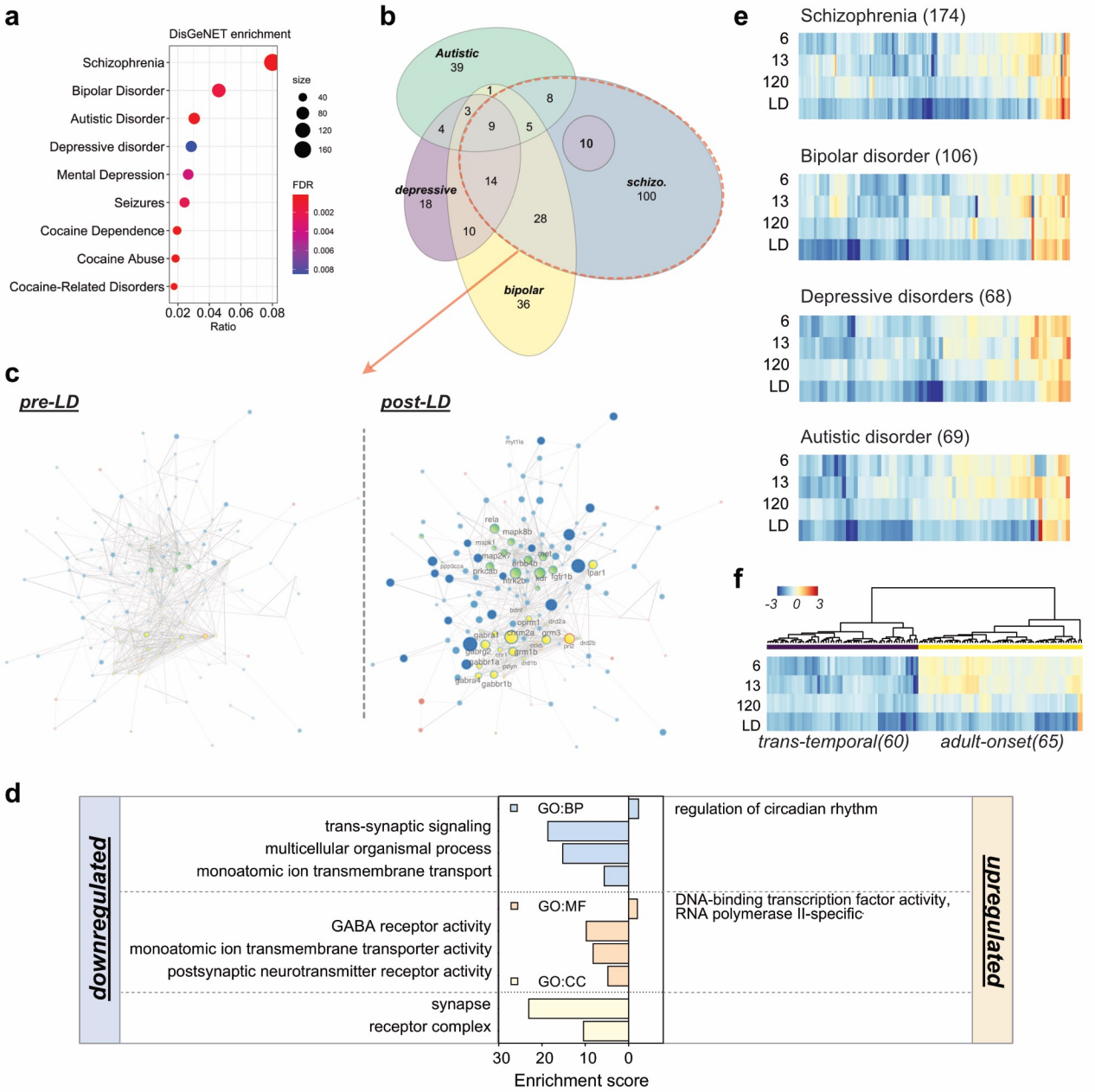

a
Natural
Simulated
Raspberry pi computer + stimulus display
Stimulus tank
Looming stimulus appears
Growing looning stimulus
Full looming stimulus
2.7 seconds
b
acute stress (LD)
wt
d120
LD+15 min
high-GC
early stage
bPAC+
high-GC
c
Cortisol (ng/g)
P < 0.0001
P = 0.9994
P < 0.0001
P = 0.0041
P < 0.0001
P = 0.4527
P = 0.0972
wt
bPAC-
bPAC+
wt (LD)
bPAC- (LD)
bPAC+ (LD)
d
nr3c1
nr3c2
crhr1
fkbp5
tsc22d3
hsd11b2
mean_CPM
wt
bPAC+
d120
LD
e
pre-LD
post-LD
wt
DEG1
LD
DEG3
bPAC+
DEG2
f
z-score
DEG1
d120:wt
LD:wt
DEG3
d120:bPAC+
LD:bPAC+
DEG2
g
adult bPAC+ DEGs
DEG1
44
18
DEG3
922
1110
DEG2
463
GC-primed
h
neuron projection devel...
regulation of localization
synaptic signaling
BP
kinase activity
metal ion transmembra...
voltage-gated cation ch...
MF
cell junction
synapse
receptor complex
CC
Enrichemnt score

Figure 4


a
DisGeNET enrichment
Schizophrenia
Bipolar Disorder
Autistic Disorder
Depressive disorder
Mental Depression
Seizures
Cocaine Dependence
Cocaine Abuse
Cocaine-Related Disorders
0.02 0.04 0.06 0.08
Ratio
size
40
80
120
160
FDR
0.002
0.004
0.006
0.008
b
Autistic
39
depressive
18
schizo.
100
bipolar
36
1
8
3
4
9
5
10
14
28
10
c
pre-LD
post-LD
e
Schizophrenia (174)
Bipolar disorder (106)
Depressive disorders (68)
Autistic disorder (69)
6
13
120
LD
f
-3 0 3
6
13
120
LD
trans-temporal(60)
adult-onset(65)

d
downregulated
upregulated
GO:BP
trans-synaptic signaling
multicellular organismal process
monoatomic ion transmembrane transport
regulation of circadian rhythm
GO:MF
GABA receptor activity
monoatomic ion transmembrane transporter activity
postsynaptic neurotransmitter receptor activity
DNA-binding transcription factor activity,
RNA polymerase II-specific
GO:CC
synapse
receptor complex
30 20 10 0
Enrichment score

Figure 5

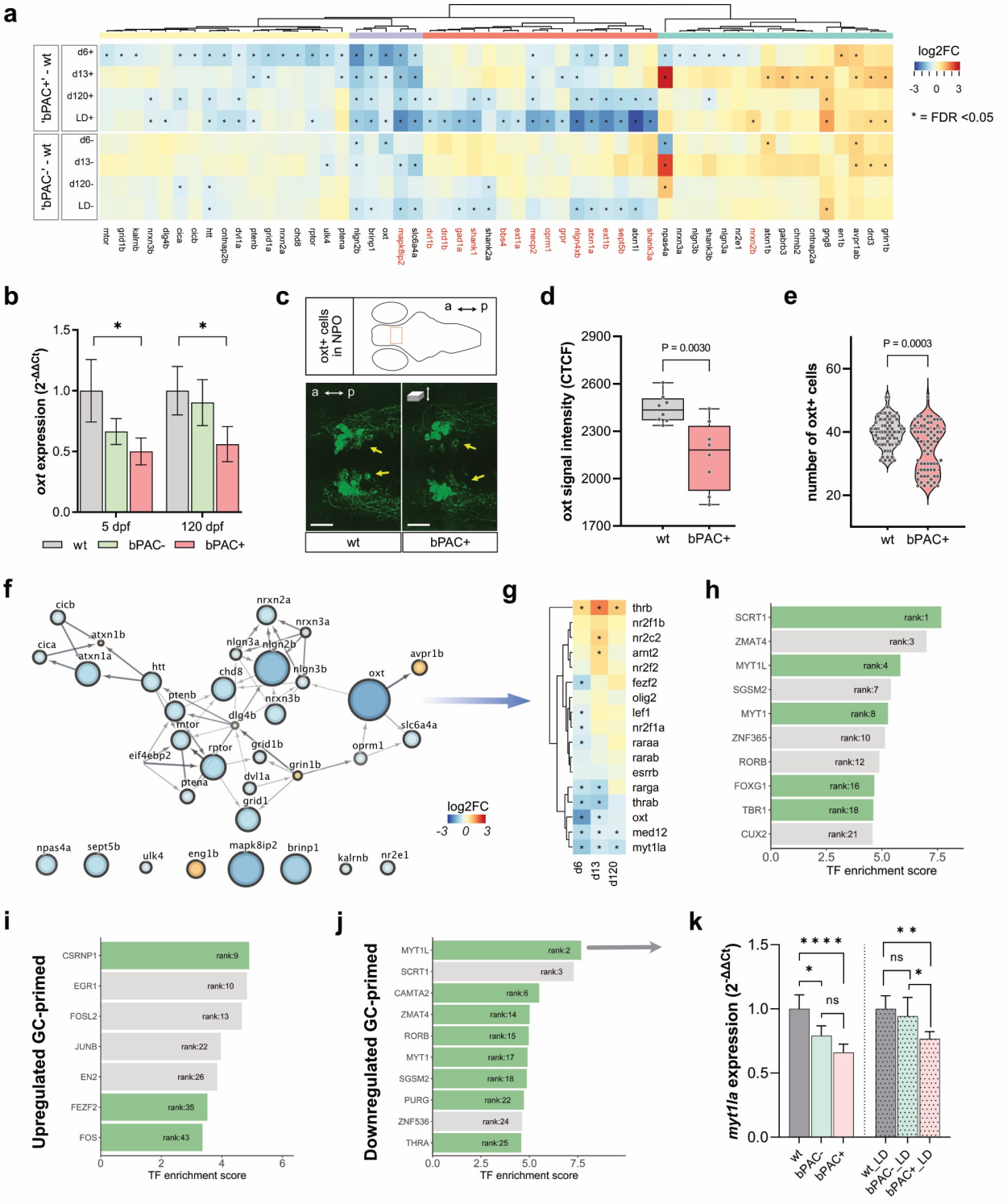

a
'bPAC+' - wt
'bPAC-' - wt
d6+
d13+
d120+
LD+
d6-
d13-
d120-
LD-
log2FC
-3 0 3
* = FDR <0.05
mtor grid1b kalrnb nrxn3b dlg4b cica cicb htt cntnap2b dvl1a ptenb grid1a nrxn2a chd8 rptor ulk4 ptena nlgn2b brinp1 oxt mapk8ip2 slc6a4a dvl1b drd1b gad1a shank1 shank2a bbs4 ext1a mecp2 oprm1 gpr nlgn4b atxn1a ext1b sept5b atxn1l shank3a npas4a nrxn3a nlgn3b shank3b nlgn3a nr2e1 nrxn2b atxn1b gabrb3 cntnap2a gng8 eng1b avpr1ab drd3 grin1b
b
oxt expression (2^-ΔΔCt)
1.5
1.0
0.5
0.0
*
*
5 dpf
120 dpf
wt
bPAC-
bPAC+
c
oxt+ cells in NPO
a ↔ p
a ↔ p
wt
bPAC+
d
oxt signal intensity (CTCF)
2900
2600
2300
2000
1700
P = 0.0030
wt
bPAC+
e
number of oxt+ cells
60
40
20
P = 0.0003
wt
bPAC+
f
cicb
cica
atxn1b
atxn1a
htt
nrxn2a
nrxn3a
nlgn3a
nlgn2b
nlgn3b
chd8
ptenb
nrxn3b
dlg4b
mtor
rptor
eif4ebp2
grid1b
grin1b
dvl1a
ptena
grid1
oxt
avpr1b
slc6a4a
oprm1
log2FC
-3 0 3
npas4a
sept5b
ulk4
eng1b
mapk8ip2
brinp1
kalrnb
nr2e1
g
thrb
nr2f1b
nr2c2
arnt2
nr2f2
fezf2
olig2
lef1
nr2f1a
raraa
rarab
esrrb
rarga
thrab
oxt
med12
myt1la
d6
d13
d120
h
SCRT1 rank:1
ZMAT4 rank:3
MYT1L rank:4
SGSM2 rank:7
MYT1 rank:8
ZNF365 rank:10
RORB rank:12
FOXG1 rank:16
TBR1 rank:18
CUX2 rank:21
0.0
2.5
5.0
7.5
TF enrichment score
i
Upregulated GC-primed
CSRNP1 rank:9
EGR1 rank:10
FOSL2 rank:13
JUNB rank:22
EN2 rank:26
FEZF2 rank:35
FOS rank:43
0
2
4
6
TF enrichment score
j
Downregulated GC-primed
MYT1L rank:2
SCRT1 rank:3
CAMTA2 rank:6
ZMAT4 rank:14
RORB rank:15
MYT1 rank:17
SGSM2 rank:18
PURG rank:22
ZNF536 rank:24
THRA rank:25
0.0
2.5
5.0
7.5
TF enrichment score
k
myt1la expression (2^-ΔΔCt)
1.5
1.0
0.5
0.0
* * * *
*
ns
* *
ns
*
wt
bPAC-
bPAC+
wt_LD
bPAC-_LD
bPAC+_LD

Figure 6