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Hippocampal brain-derived neurotrophic factor determines recruitment of anatomically connected networks after stress in diabetic mice

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Abstract

Diabetes increases adrenal steroids in humans and animal models, but potential interactions with psychological stress remain poorly understood. Diabetic rodents exhibit anxiety and reductions in hippocampal brain-derived neurotrophic factor (BDNF) expression, and these studies investigated whether loss of BDNF-driven hippocampal activity promotes anxiety and disinhibits the HPA axis. Mice with genetic obesity and diabetes (db/db) received intrahippocampal injections of lentivirus for BDNF overexpression (db/db-BDNFOE), and Wt mice received lentiviral constructs for BDNF knockdown (Wt-BDNFKD). Behavioral anxiety, and glucocorticoid responses to acute restraint were compared with mice that received a fluorescent reporter (Wt-GFP, db/db-GFP). These experiments revealed that changes in hippocampal BDNF were necessary and sufficient for behavioral anxiety and HPA axis disinhibition. To examine patterns of stress-induced regional activity, we used algorithmic detection of cFos and automated segmentation of forebrain regions to generate maps of functional covariance, which were subsequently aligned with anatomical connectivity weights from the Brain Architecture Management database. db/db-GFP mice exhibited reduced activation of the hippocampal ventral subiculum (vSub) and anterior bed nucleus of stria terminalis (aBNST), and increases in the paraventricular hypothalamus (PVH), relative to Wt-GFP. BDNFKD recapitulated this pattern in Wt mice, and BDNFOE normalized activation of the vSub>aBNST>PVH pathway in db/db mice. Analysis of forebrain activation revealed largely overlapping patterns of network disruption in db/db-GFP and Wt-BDNFKD mice, implicating BDNF-driven hippocampal activity as a determinant of stress vulnerability in both the intact and diabetic brain.

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Type 2 (insulin resistant) diabetes is accompanied by increased rates of depression and anxiety in humans (Anderson et al, 2001; Golden et al, 2008; McIntyre et al., 2010). While the mechanisms remain unknown, data from humans and rodents suggests that diabetes alters the structure and function of medial temporal lobe regions, including the hippocampus (Biessels and Reagan, 2015). Diabetes impairs hippocampal function, and the degree of impairment is correlated with depressive symptoms in humans (den Heijer et al, 2003; Bruehl et al, 2009; Ursache et al, 2012). Anxiety and hippocampal synaptic dysfunction have also been reported in rodent models of diabetes (Stranahan et al, 2008; Revsin et al, 2009; Wosiski-Kuhn et al, 2014; Guo and Lu, 2014). Given the established role of hippocampal neurons in appropriate termination of endocrine responses to stress (Herman et al, 1989), a relationship between impairment of hippocampal function and HPA axis dysregulation in diabetic individuals is likely, but has never been assessed.

Hippocampal regulation of the HPA axis is mediated by projections to the anterior bed nucleus of the stria terminalis (aBNST; Herman et al, 1989; Radley and Sawchenko, 2011). Inhibitory neurons in the aBNST reduce production of corticotrophin-releasing factor (CRF) among neurons in the paraventricular hypothalamus (PVH; Radley et al, 2009) thereby suppressing stress-induced elevations in adrenal steroids. Leptin receptor deficient db/db mice are obese and insulin resistant, with elevated levels of corticosterone, the primary glucocorticoid in rodents (Saito et al, 1983). db/db mice exhibit glucocorticoid-mediated deficits in dentate gyrus long-term potentiation (Stranahan et al, 2008), as well as reduced hippocampal BDNF expression (Stranahan et al, 2009). While reductions in BDNF may be partially mediated by the lack of functional leptin receptors (Yamada et al, 2011), we previously demonstrated that reducing corticosterone in db/db mice rescues hippocampal BDNF via leptin receptor-independent mechanisms (Stranahan et al, 2011; Wosiski-Kuhn et al, 2014). Because selective knockdown of hippocampal BDNF prolongs stress-induced HPA axis activation in normoglycemic, nonobese rats (Taliaz et al, 2011), we hypothesized that loss of BDNF-driven hippocampal activity disrupts endocrine responses to psychological stress in diabetes.

In addition to their endocrine phenotype, db/db mice exhibit deficits in connectivity between hypothalamic nuclei involved in feeding and satiety (Bouret et al, 2004). Although loss of axonal projections was selective for feeding circuits in leptin-deficient ob/ob mice, leptin receptors are widely expressed (Scott et al, 2009), and there remains the possibility that extrahypothalamic regions might exhibit differential connectivity in models of leptin deficiency. At present, there are three large-scale databases for interrogation of neuroanatomical connectivity in the rodent brain. These include the Mouse Brain Architecture Project (<http://brainarchitecture.org>) (Ng et al, 2009), the Allen Brain Institute (ABA) Mouse Connectivity Project (<http://connectivity.brain-map.org>) (Oh et al, 2014), and the Brain Architecture Management System (BAMs, <http://bams1.org>) (Bota et al, 2003). These resources integrate data from high-throughput tracing experiments and published studies to generate connectivity weights for paired regions of the rodent brain. Similar tools are emerging for functional mapping of immediate early gene (IEG) expression in the rodent brain (Ragan et al, 2012; Osten et al, 2013). These approaches utilize high-resolution imaging, automated detection of IEGs, and segmentation of regional boundaries on serial images (Kim et al, 2015; Renier et al, 2016). In this report, we examined relationships

between functional covariance and connectivity weights between regions of the adult mouse forebrain. To determine whether reductions in hippocampal BDNF disrupt network activity, we measured corticosteroid responses to restraint stress and forebrain IEG expression in db/db mice. After characterizing genotype differences, we overexpressed hippocampal BDNF in db/db mice and knocked down BDNF in Wt mice using lentiviral vectors. By comparing IEG induction after stress in db/db and Wt mice with intact or impaired BDNF expression, we addressed the consequences of reduced hippocampal BDNF for network recruitment in obesity and diabetes.

Methods

Animals and experimental design

Male C57Bl6J (Wt) mice and db/db mice on the C57Bl6J background were obtained from Jackson Laboratories (Bar Harbor, ME, USA) at 6wk old. In the first set of experiments, mice were acclimated to the facility for 1wk before behavioral testing, restraint stress, and transcardial perfusion as described in Fig.1A and in Supplementary Methods. In the second set of experiments, Wt and db/db mice received stereotaxic injections of lentiviral vectors followed by behavior testing 11d after surgery. After testing, mice received a single episode of restraint stress before transcardial perfusion with paraformaldehyde (n=7) or phosphate-buffered saline (PBS; n=4–6). Brains from mice perfused with PBS were rapidly extracted from the skull and the right hippocampus was excised and frozen for BDNF ELISA. Detailed methodology for restraint stress, blood sampling, and ELISAs for BDNF and corticosterone are provided in the Supplementary Methods. Lentiviral vectors were also generated using published protocols (de Almeida et al, 2001) and are described in Supplementary Methods. All experiments followed NIH Guidelines and were approved by the Institutional Animal Care and Use Committee at Augusta University.

Behavioral testing

Testing in the open field, elevated plus maze (EPM) and light/dark box was carried out during the first half of the dark phase (18:00–21:00). Open field testing followed previously published methods (Stranahan et al, 2009), with minor modifications to accommodate video tracking rather than optical beam breaks. The EPM was set 50cm above the floor and consisted of two perpendicular arms (45cm long × 10cm wide), with walls (40cm high) surrounding the enclosed arms. EPM testing consisted of 10min exploration under red light illumination. Test sessions were captured on digital video for offline analysis using Tracker (freely available at physlets.org/tracker). Testing in the light/dark box was conducted under white light in an adjacent room, with a ten-minute exploration session recorded on digital video and analyzed offline using Tracker freeware as described above. For the novelty-suppressed feeding and gustatory neophobia tasks, testing was conducted under red light illumination after 2hr food deprivation (food withheld 18:00–20:00; water available ad libitum). In the novelty-suppressed feeding test, mice were transferred to an arena (60cm long × 45cm wide) with 30cm high black walls. A single pellet of rodent chow (Teklad Diet #8656; Teklad Diets, Madison, WI) was placed in the middle of the arena and consumption was recorded after 10min. In the gustatory neophobia test, a novel food (Froot Loop;

General Mills, Minneapolis, MN) was placed in the home cage, with consumption recorded after 10min.

Manual stereology and serial section tiled immunofluorescence (ssTIF)

Immunofluorescence detection of cFos was performed using a slide-mounted protocol and methods for stereological quantification are described in Supplementary Methods. For ssTIF, montage images of cFos and DAPI were captured at 100X total magnification on a Zeiss MTB microscope with a motorized stage. After manual plane-of-focus calibration, tiles were acquired and montaged in Microlucida software (Microbrightfield, Williston, VT). Serial section images were aligned using elastic stack alignment in Fiji software (<https://fiji.sc/>). Aligned images were subject to algorithmic background subtraction with constant threshold parameters and cFos-labeled nuclei were automatically detected based circularity and size criteria. For regional segmentation, images were registered onto the Allen Brain Atlas Common Coordinate Framework (CCF; <http://download.alleninstitute.org>). To measure regional volumes in db/db mice, the CCF template was transformed onto tiled images of DAPI staining ('reverse registration') and the resulting 2D areas were converted to volumes based on the principle of Cavalieri (Table S1). For analysis, cell counts were normalized to regional volumes and expressed as densities for statistical comparison.

Statistics

For details of genotype comparisons and single-endpoint statistical analysis after BDNF manipulation, see Supplementary Methods. For the ssTIF dataset, regional volumes were compared across conditions using ANOVA. Post hoc comparisons were made relative to Wt-GFP by computing the t-distribution and associated p-values for each region. The false discovery rate (FDR)-adjusted q-values were computed from p-values to correct for multiple comparisons, as shown (Table S2). When significant differences in regional volume were observed, the structure was excluded from subsequent analysis. cFos densities in regions with no evident structural atrophy were normalized by z-transformation and compared across groups using one-way ANOVA with planned post hoc comparisons. FDR-adjusted q-values were calculated from the relevant p-values for each comparison, as described (Stranahan et al, 2010; Stranahan et al, 2012). Principal component analysis (PCA) was used to identify anatomical regions driving variability between experimental conditions. The PCA was conducted blind, with each sample plotted as a single point on a 3-dimensional graph. The XYZ axes represent the proportion of variance explained by the top (3) principal components, which were orthogonal within the range of the ssTIF dataset. All analyses of the ssTIF dataset were carried out in JMP version 13.0 (SAS, Cary, NC).

Generation of connectivity matrix and connectivity/coactivation analysis

The Brain Architecture Management System (BAMs; <http://bams1.org>) generates ranked measures of projection strength from >16,000 primary datasets (Bota et al, 2015). Connectivity weights in BAMs are represented as ordinal values ranging from absent to very strong, excluding intra-regional or local connections, and the details of content mining and updating are outlined in Supplementary Methods. To examine relationships between connectivity and regional activation, hierarchical cluster analysis was applied to cFos+ counts on a per-animal basis. Standardized centroid distance matrices were generated from

cell counts in paired regions and the stacked distance matrices were aligned with corresponding ordinal values from BAMs (Fig.S2). Within-group relationships between clustering and connectivity were analyzed using logistical regression and Chi-squared. Standardized clustering generates normally distributed measures of regional covariance that can be compared across groups using parametric statistics. Standardized cluster distances at each level of connectivity were analyzed by one-way ANOVA with planned post hoc comparisons in JMP version 13.0 (SAS, Cary, NC).

Results

Behavioral anxiety and impaired negative feedback on the HPA axis in diabetic mice

db/db mice exhibited more anxiety than Wt mice, but locomotor hypoactivity complicates the interpretation of exploratory tasks in this model. To promote exploration, db/db and Wt mice were tested at the onset of the dark phase under red light illumination (Fig.1A). Consistent with previous reports (Stranahan et al, 2009; Guo and Lu, 2014), db/db mice spent proportionally less time exploring the center of the arena (Fig.1B). Similar patterns were observed in the EPM, where preferential exploration of the enclosed arm was observed after normalizing to total distance in db/db mice (Fig.1C; $F_{1,22}=7.86$, $p<0.01$). In the light/dark box, db/db mice spent less time in the light side (Fig.1D; $t_{14}=2.62$, $p<0.05$), but also crossed between the light and dark sides less frequently ($t_{14}=3.09$, $p<0.01$). The duration of individual light side exploratory episodes was significantly shorter in db/db mice (Fig.1D; $t_{14}=2.73$, $p<0.05$), indicative of increased anxiety as well as reduced activity.

In addition to their anxiogenic phenotype, db/db mice are hyperphagic, relative to Wt mice (Cox and Powley, 1977). To examine approach/avoidance conflict in response to food, we tested db/db and Wt mice in the novelty-suppressed feeding and gustatory neophobia tasks. For the novelty-suppressed feeding paradigm, mice were fasted for 2hr before testing in a novel arena with a pellet of standard chow (Teklad Diet #8656) in the center. These experiments revealed that db/db mice waited longer to approach a familiar food in a novel environment (Fig.1E; $t_{10}=4.31$, $p<0.05$), and consumed less food than Wt mice ($t_{10}=2.86$, $p<0.05$). In the gustatory neophobia task, mice were fasted as outlined above before introduction of an unfamiliar food (Froot Loop) into the home cage. db/db mice also took more time to approach the unfamiliar food in a familiar environment (Fig.1E; $t_{10}=3.72$, $p<0.05$), and consumed less of the unfamiliar food than Wt mice ($t_{10}=3.42$, $p<0.01$), indicative of anxiogenic responses to environmental and gustatory novelty.

Impaired HPA negative feedback in diabetic mice

db/db mice exhibit elevated basal corticosterone levels, but glucocorticoid responses to psychological stress had never been characterized in this model. To this end, we measured serum corticosterone levels before and after acute restraint stress in Wt and db/db mice. db/db mice exhibit elevated basal corticosterone levels, and were unable to recover to pre-stress levels after restraint (Fig.1F; $F_{1,20}=2.54$, $p<0.05$). Quantification of cFos+ cells in the PVH revealed increased activation in db/db mice (Fig.1G; $t_{10}=3.58$, $p<0.01$). Because there was no change in total neuron number in the anterior PVH of db/db mice (Fig.1G), we

concluded that diabetic mice exhibit prolonged HPA axis activation following psychological stress.

We next quantified cFos+ nuclei to determine whether reduced aBNST activation might be associated with increased PVH activity in diabetic mice (Fig.2A-B). db/db mice exhibit reduced cFos+ cell numbers in the anterolateral (alBNST; $t_{10}=5.44$, $p<0.01$) and anteromedial regions of the BNST (amBNST; $t_{10}=8.28$, $p<0.01$). There was no effect of genotype on cFos+ cell numbers in the ventral aBNST (Fig.2A, ns). GABAergic neurons in the aBNST send inputs to the PVH, and this relay promotes HPA negative feedback (Radley and Sawchenko, 2011). We therefore investigated whether db/db mice exhibit reduced activation of aBNST inhibitory neurons after acute stress. db/db mice had fewer cFos+/GAD67+ cells (Fig.2C-E; $t_{10}=7.75$, $p<0.01$), consistent with reduced activity among GABAergic neurons in the aBNST. Quantification of total GAD67-labeled cell number revealed no differences between db/db and Wt mice (Fig.2C-D; ns), and there was also no effect of genotype on total neuron number (mean±sem: Wt=18,566±4,298; db/db=19,279±5,405) or regional volumes (mm³, mean±sem: Wt=0.07±0.02; db/db=0.08±0.03). Taken together, these observations indicate that the cellularity and structure of the aBNST was intact.

Hippocampal neurons project to the aBNST, and these projections mediate hippocampal negative feedback on the HPA axis. To determine whether stress-induced activation of hippocampal neurons might be disrupted in db/db mice, we quantified cFos+ nuclei in the dentate gyrus (DG), CA3, CA1, and subiculum (Fig.2F-G). db/db mice had significantly fewer cFos+ cells in the DG ($t_{10}=4.96$, $p<0.01$) and subiculum ($t_{10}=2.54$, $p<0.05$, Fig.2F-G). Quantification of total neuron number (Fig.S1A) and the volume of individual hippocampal subfields (Fig.S1B) revealed no evidence of regional atrophy, and are consistent with reduced activation, rather than neuronal loss or impaired hippocampal development in db/db mice.

Overexpression and knockdown of hippocampal brain-derived neurotrophic factor

To determine whether reductions in hippocampal BDNF promote anxiety in diabetes, we used lentiviral vectors to overexpress BDNF in db/db mice (db/db-BDNFOE) and knock down BDNF in Wt (Wt-BDNFKD). Parallel groups of Wt and db/db mice received stereotaxic injections of reporter construct (GFP; (Wt-GFP, db/db-GFP; Fig.3A-B). The overexpression vector restored hippocampal BDNF protein levels in db/db mice (Fig.3C), and the knockdown vector reduced BDNF in Wt mice ($F_{2,17}=4.75$, $p=0.02$). There was no effect of BDNF knockdown on body weight in Wt mice, and BDNF overexpression had no effect on obesity in db/db mice (Fig.S2). Analysis of behavioral anxiety in the EPM revealed that db/db-BDNFOE mice exhibited increased open arm exploration, relative to db/db-GFP mice ($F_{3,44}=14.84$, $p=0.001$; Fig.3D). This effect was not due to changes in locomotor activity, as total distance was reduced in both groups of db/db mice, relative to Wt-GFP (Fig. 3D; $F_{3,44}=12.33$, $p<0.01$). Wt-BDNFKD mice exhibited reduced open arm exploration without changes in total distance (Fig.3D), indicating that reductions in BDNF promote behavioral anxiety under these conditions. Similar effects were observed in the light/dark box, where BDNF knockdown reduced exploration of the light side in Wt mice (Fig.3E),

and BDNF overexpression increased time in the light side in db/db mice ($F_{3,19}=5.39$, $p<0.01$), relative to genotype-matched controls that received reporter constructs. Because both groups of db/db mice crossed between the two sides less frequently than Wt (Fig.3E, $F_{3,19}=5.07$, $p<0.01$), we quantified the average duration of exploratory episodes in the light side of the arena (seconds per exploratory bout/n bouts). Hippocampal BDNF overexpression normalized the duration of individual light side exploratory episodes in db/db mice (Fig.3E), and reduced the average duration of light side explorations in Wt-BDNFKD mice ($F_{3,19}=14.88$, $p<0.001$), suggesting that reductions in hippocampal BDNF are necessary and sufficient for behavioral anxiety in this setting.

We next quantified corticosterone levels after acute restraint stress. Both groups of db/db mice had elevated basal corticosterone, but db/db-BDNFOE mice recovered to pre-stress levels 2hr later (Fig.3F; $F_{3,34}=9.59$, $p<0.01$). In Wt mice, BDNFKD prolonged stress-induced elevations in corticosterone without affecting basal corticosterone levels (Fig.3F; $F_{1,41}=9.28$, $p<0.01$). Changes in stress-induced corticosterone levels were accompanied by alterations in the number of cFos+ nuclei in the PVH (Fig.3G-H). In Wt mice, BDNFKD increased the number of cFos-labeled cells in the anterior PVH ($F_{3,31}=9.79$, $p<0.01$). In db/db mice, BDNFOE reduced the number of cFos+ nuclei in the PVH (Fig.3G-H). These data indicate that hippocampal BDNF is required for negative feedback on the HPA axis, but is dispensable for basal corticosterone synthesis.

Hippocampal BDNF regulates activation of the anterior BNST

To investigate the role of hippocampal BDNF in stress-induced neural activation, we used serial section tiled immunofluorescence (ssTIF), algorithmic detection of cFos+ nuclei, and automated segmentation of regional boundaries, as shown (Fig.4A). These experiments revealed that BDNFKD reduced cFos induction in the hippocampal DG of Wt mice after restraint stress (Fig.4B; see Table S2 for statistics). Loss of stress-induced cFos induction in db/db mice was eliminated by BDNF overexpression (Fig.4B, Table S2). The consequences of BDNFKD diverged from those observed in db/db-GFP mice in hippocampal area CA3, where Wt-BDNFKD mice exhibited reductions relative to Wt-GFP (Fig.4B, Table S2). BDNFKD also reduced cFos+ cells in hippocampal area CA2, and similar reductions were observed in db/db-GFP mice (Fig.4B, Table S2). Reductions in cFos induction after stress in CA2 were BDNF-mediated, as db/db-BDNFOE mice had similar cFos+ cell densities relative to Wt-GFP (Fig.4B, Table S2).

There were no changes in cFos induction in the medial and lateral entorhinal regions, but selective alterations were present in the ventral subiculum (SUBv). Wt-BDNFKD mice exhibited reductions in cFos+ cell densities, relative to Wt-GFP (Fig.4B, Table S2). The reductions observed in Wt-BDNFKD mice were comparable to db/db-GFP mice (Fig.4B, Table S2). db/db-BDNFOE mice exhibited SUBv cFos induction that was comparable to Wt-GFP, indicating that DG BDNF determines activation of hippocampal and retrohippocampal regions after stress.

Analysis of pallidal regions revealed reduced activation of the aBNST and an unexpected effect of genotype on cFos induction in the substantia innominata (SI). The SI exhibited significant, BDNF-independent reductions in cFos expression in db/db mice (Fig.4C, Table

S2). In the aBNST, BDNF knockdown reduced cFos induction in Wt mice (Fig.4C, Table S2). db/db-GFP mice also exhibited reductions in cFos+ cells that were eliminated by BDNFOE (Fig.4C, Table S2). Changes in cFos in the hippocampal DG and aBNST were upheld by quantification with traditional stereological methods (Fig.4D). Manual cell counts were performed on sections previously quantified by ssTIF and stereological estimates were compared with the number of cells detected using circularity and brightness criteria. This approach revealed significant correlations between cell counts generated using ssTIF and manual counting in the hippocampal DG (Fig.4D; $R^2=0.72$, $p<0.01$) and aBNST (Fig.4D; $R^2=0.68$, $p<0.01$). Statistically significant correlations were not driven by group differences, as significant within-group correlations were also observed between the two measures (Pearson's r ; Wt-GFP, $r=0.54$, $p<0.01$; Wt-BDNFKD, $r=0.32$, $p<0.05$; db/db-GFP, $r=0.42$, $p<0.01$; db/db-BDNFOE, $r=0.60$, $p<0.01$).

Hippocampal BDNF regulates regional activity after stress

After validating the changes observed in the hippocampus and aBNST by manual stereology, we examined broader regulation of stress-induced cFos induction following BDNF overexpression or knockdown. In cortex, db/db-GFP and Wt-BDNFKD mice had fewer cFos+ cells in the prelimbic (PL) and infralimbic areas, relative to Wt-GFP (ILA; Fig.4E, see Table S2 for statistics). BDNF-dependent increases in cFos+ cells were also observed in the dorsal anterior cingulate cortex (ACAd) of db/db-GFP mice (Fig.4E, Table S2). Although increases in ACAd activation were reversed by BDNFOE in db/db mice, this effect was not recapitulated by BDNF knockdown in Wt (Fig.4E, Table S2). Changes in cFos were regionally differentiated along the dorsoventral axis of the anterior cingulate area, as comparable increases in cFos+ cells were observed in the ventral anterior cingulate cortex (ACAv) of db/db mice injected with either vector (Fig.4E, Table S2). In the anterior insula (AI), hippocampal BDNF overexpression reduced cFos induction in db/db mice, and this reduction was not observed in db/db-GFP or Wt-BDNFKD (Fig.4E, Table S2).

Changes in hippocampal BDNF also regulated stress-induced cFos activation in widespread regions of the extended amygdala (Fig.4F-G). Specifically, reductions in hippocampal BDNF due to diabetes or knockdown increased cFos+ cells in the basolateral and basomedial amygdala (BLA, BMA; Fig.4F, see Table S2 for statistics). BDNFKD reduced cFos+ cell densities in the posterior amygdala (PA) of Wt mice, with no detectable alterations in db/db mice injected with either vector (Fig.4F, Table S2). The overlapping effects of hippocampal BDNFKD and leptin receptor deficiency were also evident in the central (CEA) and medial amygdala (MEA) (Fig.4G, Table S2).

Genetic obesity and diabetes also disrupted cFos induction in olfactory regions, including the anterior cortical amygdalar area (COAa) and the piriform-amygdalar transition area (PAA; Fig.4H, Table S2). These effects were eliminated by BDNFOE, but were not recapitulated by BDNF knockdown in Wt mice (Fig.4H, Table S2). There were also unexpected similarities between the effects of BDNFOE in db/db mice and the consequences of BDNFKD in Wt mice on cFos+ cells in the piriform cortex (PIR), suggestive of a nonlinear interaction between hippocampal BDNF expression and stress-induced activation

in the PIR (Fig.4H, Table S2). Overall, these data revealed overlapping patterns of activation in db/db-GFP mice and Wt-BDNFKD mice (Fig.4I).

Relationships between anatomical innervation and regional activation after acute stress

To examine patterns of regional activity after acute stress, we performed principal components analysis (PCA) on cFos counts, followed by hierarchical cluster analysis (Fig. 5A). In the PCA dataset, three sets of regional vectors accounted for nearly half of the variance (46.6%) between experimental conditions (Fig.5B). Seven of the eight regional vectors within principal component 1 (PC1) were BNST nuclei (Table S3). Multiple regions of the extended amygdala were identified in PC2, while PC3 vectors included the septal complex and frontal cortical regions (Table S3).

Hierarchical clustering revealed greater functional covariance between paired regions in Wt-GFP, relative to db/db-GFP and Wt-BDNFKD animals (Fig.5C-D). Specifically, the absolute difference in cFos counts between paired regions was significantly greater in db/db-GFP mice, relative to Wt-GFP mice (Fig.5C; $F_{3,31}=9.76$, $p<0.01$). Loss of inter-regional covariance was BDNF-dependent, as db/db-BDNFOE mice were indistinguishable from Wt-GFP, while Wt-BDNFKD animals exhibited increases that were similar to db/db-GFP (Fig. 5C). Disruption of functional covariance was also evident based on inter-regional correlations, with significantly more non-correlated region pairs in db/db-GFP and Wt-BDNFKD mice (Fig.5D).

To investigate whether overexpression or knockdown of hippocampal BDNF might regulate activity within anatomically connected networks, standardized distance matrices from the hierarchical clustering dataset were aligned with the corresponding connectivity weights from the BAMs database (Fig.5E, Fig.S3). The 97×97 matrix examined in this report contained 9,409 region pairs, and the BAMs database included data for 62% of these projections. The connections included in the BAMs dataset were normally distributed across the ordinal connectivity weights used to reflect projection strength (Fig.5F). Within-group analysis of connectivity $\times$ clustering relationships revealed that regions with stronger anatomical connectivity exhibited greater functional covariance in Wt-GFP mice (Fig.5G, within-group $\chi^2=7.14$, $p<0.01$). In db/db-GFP mice, the connectivity $\times$ clustering relationship was lost, based on within-group analysis (Fig.5G, within-group $\chi^2=2.28$, ns), and on across-group comparison of standardized cluster distances at each level of connectivity (Fig.5H; $F_{3,27}=9.42$, $p<0.01$; post hoc comparison with Wt-GFP, $p<0.05$ at moderate-strong, strong, and very strong). Relationships between connectivity and regional covariance were reduced but not eliminated in Wt-BDNFKD mice (Fig.5G=H, within-group $\chi^2=4.82$, $p<0.05$; $F_{3,27}=9.42$, $p<0.01$; post hoc comparison with Wt-GFP, $p<0.05$ at moderate-strong, strong, and very strong). BDNF overexpression normalized connectivity $\times$ clustering relationships in db/db mice (Fig.5G-H, within-group $\chi^2=0.95$, RMSE <0.05 ; post hoc comparison with Wt-GFP, ns), suggesting that hippocampal BDNF determines downstream recruitment of anatomically connected regions after stress.

Leptin deficient mice exhibit deficits in connectivity between hypothalamic feeding nuclei (Bouret et al, 2004). Although regions exhibiting structural atrophy in db/db-GFP mice were excluded from the current dataset, we cannot rule out the possibility that differences in

anatomical innervation may still be present among structurally intact areas that have not yet been characterized with respect to their connections. To examine the consequences of reduced anatomical connectivity for cFos induction, we relied on published reports of atrophied projections between the hypothalamic arcuate nucleus (ARH) and dorsomedial (DMH), and between the ARH and PVH in leptin deficient mice (Bouret et al, 2004). This same set of studies determined that connectivity between the BNSTpr and PVH, and between the PVH and DMH, was intact in ob/ob mice. Examination of regional covariance between the above areas in Wt-GFP and db/db-GFP mice revealed an inverse relationship between clustering and connectivity (Fig.S4). Cluster distances between cFos+ cell counts in areas with reduced connectivity were significantly greater in db/db-GFP mice, relative to Wt-GFP ($F_{1,24}=3.98$, $p=0.027$; Fig.S4). Although this set of regions represents only a small fraction of the rodent brain connectome, the results suggest that it may eventually become possible to interrogate the functional architecture of neural circuits using similar methodology.

Discussion

The outcome of these experiments suggests the existence of an anatomically weighted threat detection network, with hippocampal activation as a critical node determining patterns of circuit recruitment. In normal mice, selective reductions in hippocampal BDNF were associated with widespread changes in cFos induction after acute stress and loss of anatomically-driven patterns of activity. In db/db mice, reinstatement of hippocampal BDNF normalized stress-induced immediate early gene induction and restored the relationship between functional co-activation and connectivity. These BDNF-mediated changes in circuit recruitment regulate negative feedback on the HPA axis, because BDNF knockdown prolonged glucocorticoid responses to stress in Wt, while overexpression enhanced recovery in db/db mice. Given that distortion of stress-induced network recruitment in db/db mice was eliminated by BDNF overexpression, we conclude that metabolic stress in diabetes interacts with psychological stress by regulating hippocampal BDNF.

Compilation of the rodent neuroanatomical connectome has been ongoing for over ten years, and the current iteration of the BAMs database includes over 75,000 connections from original and published research reports (Bota et al, 2003; Bota et al, 2015). Computational pipelines for functional mapping of brain activity have also been developed (Ragan et al, 2012; Osten et al, 2013), but integrating measures of connectivity into functional mapping studies presents certain challenges. Adeno-associated virus (AAV) tracing of neural projections has previously been used to investigate circuit-specific cFos induction after social behavior in mice (Kim et al, 2015), and to investigate differences in connectivity between mPFC neurons activated by aversive or rewarding experiences (Ye et al, 2016). When combined with promoter-specific cre expression, AAV tracing has the advantage of neurochemical specificity, although the interpretation of relative projection strength is relatively limited. Ye and colleagues (2016) applied algorithms from diffusion tensor imaging to generate continuous measures of projection strength after AAV tracing. This approach offers quantitative accuracy, but stereotaxic delivery of AAV constrains the number of potential seed regions in a given animal. In this report, we examined relationships between regional covariance and connectivity weights based on aggregate data from tract

tracing studies in rodents. Functional covariance, as reflected by cFos induction, was strongest between highly interconnected regions in Wt-GFP mice, dampened by BDNFKD in Wt, and absent in db/db-GFP mice. The observed relationship between function and connectivity is exclusively correlational at present, and future studies will be required to determine how inactivation or denervation of specific connections degrades functional covariance.

These studies focused on forebrain regions that regulate stress and anxiety, and we initially considered whether the absence of functional leptin receptors might impact the development of the hippocampus, BNST, and PVH. Changes in cFos were not explained by changes in total neuron number in these areas, suggesting that the cellular integrity of this stress-regulatory circuit was unaffected by leptin receptor mutation. We also observed comparable numbers of GAD67-expressing cells in the aBNST, suggesting that the local population of inhibitory neurons was intact. However, we cannot rule out the possibility that specific interneuron subsets in the BNST might be altered in db/db mice. Somatostatin-expressing interneurons predominate in the dorsolateral BNST (Nguyen et al, 2016), where reductions in GAD67/cFos+ cells were observed in db/db mice in the current studies. Calretinin-positive interneurons are also enriched in the BNST, while fast-spiking parvalbumin interneurons are scarce (Nguyen et al, 2016). Given that the firing characteristics vary between different interneuron subsets, the results observed in the initial comparisons between Wt and db/db mice might have arisen due to developmental effects of leptin receptor deficiency on BNST interneuron subsets. However, this interpretation is unlikely given the reinstatement of cFos induction patterns observed after overexpressing hippocampal BDNF in db/db mice. Because overexpressing hippocampal BDNF in adulthood would be unlikely to correct possible deficits in the development of BNST interneuron subsets in db/db mice, it is likely that BDNF-driven activation of hippocampal afferents to the BNST normalizes feed-forward inhibitory control over the HPA axis.

Elegant anatomical studies have demonstrated that BNST GABAergic neurons receive inputs from the SUBv and PL (Herman et al, 1989; Radley et al, 2009). Ablation of either region disinhibits the HPA axis, resulting in prolonged endocrine responses to psychological stress (Radley and Sawchenko, 2011). In the current studies, Wt-BDNFKD mice exhibited comparable reductions in PL activation, relative to db/db-GFP mice. The infralimbic area (ILA) exhibited similar patterns, although there was a stepwise reduction in ILA cFos+ cells in Wt-BDNFKD and db/db-GFP mice. Although similar outcomes were observed in the PL and ILA, the differences in neuroanatomical connectivity and function between these regions (Radley et al, 2009) suggest that distinct mechanisms may be driving changes in recruitment. Neurons in the PL and ILA express type II glucocorticoid receptors, which have lower affinity for corticosterone and are occupied after stress and during the circadian acrophase (Joels and Baram, 2009). Chronic stress or protracted administration of exogenous corticosterone reduces dendritic spine density in both regions (Radley et al, 2006; Goldwater et al, 2009; Anderson et al, 2016). Because prelimbic neurons regulate and respond to glucocorticoids, it is possible that the PLA may be an additional node where intrinsic changes contribute to HPA axis disinhibition. Given that infralimbic neurons respond to glucocorticoids without regulating glucocorticoid levels (Radley et al, 2009),

changes in cFos induction may be secondary to protracted increases in corticosterone in the ILA.

BDNF is not a classical IEG, but its expression is activity-dependent (Pruunsild et al, 2011) and activity-dependent increases in BDNF are required for memory formation (Bambah-Mukku et al, 2014). We and others have shown that db/db mice exhibit reductions in hippocampal BDNF and behavioral anxiety (Stranahan et al, 2009; Guo and Lu, 2014) along with deficits in learning and memory (Li et al, 2002; Wosiski-Kuhn et al, 2014). Although deficits in memory and BDNF expression are alleviated by reducing glucocorticoids (Stranahan et al, 2008; Revsin et al, 2009; Stranahan et al, 2011), the potential for reciprocal regulation of corticosteroids by hippocampal BDNF has never been addressed previously in any model of diabetes. The current study suggests that reductions in hippocampal BDNF impair recovery to pre-stress corticosterone levels in db/db mice without influencing basal glucocorticoid hypersecretion. Previous reports indicate that selective reductions in hippocampal BDNF prolong stress-induced elevations in corticosterone in nonobese, nondiabetic rodents (Taliaz et al, 2011), and these findings were replicated by BDNF knockdown in Wt mice in the current dataset. These results are consistent with an obligatory role for hippocampal BDNF in HPA negative feedback, but the implications for behavior are less clear, given that anxiety was detected prior to restraint stress. Hippocampal BDNF is required for the behavioral effects of antidepressants in rodent models of chronic stress (Björkholm and Monteggia, 2016), but there is some heterogeneity in the literature on basal anxiety following selective manipulations of dentate gyrus BDNF in nonstressed animals. Dentate gyrus-specific deletion of BDNF did not promote anxiety in mice (Adachi et al, 2008), but was reported to increase anxiety in rats (Taliaz et al, 2010). Although we observed increased anxiety following BDNF knockdown in Wt mice, this effect was observed during the dark cycle, when locomotor activity is highest in nocturnal rodents (Stranahan et al, 2009). Given that exploratory behavior and hippocampal BDNF expression both vary over the circadian cycle (Cirelli and Tononi, 2000), it is possible that changes in anxiety may be more prominent against a background of heightened activity and increased endogenous BDNF expression in control animals during the dark phase.

Alternatively, if hippocampal BDNF alters regional activation during testing in a similar manner to that observed after restraint, then these changes may favor exploration over threat assessment. In rodents, exploratory behavior is thought to reflect the balance between appetitive drive favoring novelty, and the aversive consequences of potential predation (Fonio et al, 2006). Sensory information is updated and integrated in an ongoing manner throughout exploratory behavior, and neurons in the hippocampal dentate gyrus were recently shown to link emotional valence with environmental features (Redondo et al, 2014). Given that db/db mice exhibit impaired dentate gyrus synaptic plasticity (Stranahan et al, 2008; Wosiski-Kuhn et al, 2014), a potential rescue following BDNF overexpression could normalize the underlying mechanisms for learned safety during behavioral testing.

Addressing interactions between diabetes, BDNF, and glucocorticoids represents a complex problem that would be more amenable to investigation in models of diet-induced obesity. db/db mice begin to exhibit increased corticosterone levels at approximately six weeks of age, with a parallel timecourse for the development of insulin resistance (Saito and Bray,

1983). By contrast, rodents with diet-induced obesity and insulin resistance exhibit minimal or no elevations in basal corticosterone, although disruption of HPA axis negative feedback has been reported (Tannenbaum et al, 1997; Auvinen et al, 2011; McNeilly et al, 2015). Published findings on anxiety and depression-like behavior in rodents with dietary obesity are also highly variable, with reports of anxiolytic (McNeilly et al, 2015), anxiogenic (Aslani et al, 2015; Almeida-Suhett et al, 2017), or no effect of obesogenic diets (Hsu et al, 2015). Consumption of palatable foods after acute restraint attenuates behavioral anxiety in normoglycemic, nonobese rodents (Ulrich-Lai et al, 2010), suggesting that changes in anxiety with obesogenic diets may be unmasked following exposure to psychological stress. Addressing interactions between diabetes and stress vulnerability will be challenging, but pursuing mechanisms for this relationship may uncover population-specific treatment strategies for individuals with comorbid diabetes and depression. Given that both depression and diabetes are clinically heterogeneous, with variable responses to different therapeutics, understanding the intersection between these conditions may refine existing approaches to treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. db/db mice exhibit anxiety and impaired termination of stress-induced elevations in glucocorticoids.

(A), Experiment schematic. Abbreviations: OF, open field; EPM, elevated plus maze; L/D, light/dark box; NSF, novelty-suppressed feeding; GN, gustatory neophobia. (B), Mice with genetic obesity and diabetes (db/db) exhibit reduced locomotion in both the center and periphery of the open field (left). Normalization to total distance (right) revealed significantly reduced exploration of the center of the arena in db/db mice (n=15 per genotype). (C), db/db mice also exhibit hypoactivity in the elevated plus maze (left), and normalization to total distance revealed preferential reductions in open arm exploration (right; Wt, n=12; db/db, n=15). (D), db/db mice spent less time exploring the light side (left), but also crossed between the two sides less frequently (middle). The average duration of exploratory bouts in the light side was significantly reduced in db/db mice (right; n=15 per genotype). (E) db/db mice waited longer to approach a familiar food in a novel environment (NSF; left), and also had longer approach latencies when a novel food was introduced into the home cage (GN). db/db mice also ate less when presented with a familiar food in a novel arena (NSF; right), and when presented with a novel food in the home cage (right; Wt, n=12; db/db, n=11). (F), Analysis of stress-induced elevations in corticosterone revealed impaired recovery after 30min restraint stress in db/db mice (n=15 per genotype). (F), Stereological quantification of cFos+ cells in the hypothalamic paraventricular nucleus revealed increased activation without reductions in total neuron number in db/db mice (n=8 per genotype). For all graphs, error bars represent the sem and asterisk (*) denotes statistical significance at $p < 0.05$ following ANOVA, repeated-measures ANOVA, or t-tests.

Figure 2. Anatomical evidence for loss of feedforward inhibition in stress-regulatory regions in db/db mice.

(A), Stereological quantification of cFos in the anterior bed nucleus of the stria terminalis (BNST) revealed reduced activation of the dorsolateral (dl) and dorsomedial (dm) regions after stress in db/db mice. (B), Micrographs depict representative images of cFos immunoreactivity in Wt and db/db mice. For panels (A-B), nomenclature (dl, dm, v) refers to anatomical position; see Methods for regional parcellation of the BNST. (C), Images show immunoperoxidase staining for GAD67 in dorsal regions of the anterior BNST. (D), No effect of leptin receptor mutation on GAD67+ cells or total neuron number in the dorsal nuclei of the anterior BNST. (E), Representative images of cFos and GAD67 immunofluorescence in the dorsal nuclei of the anterior BNST. (F), Stereological quantification of cFos induction in hippocampal subfields revealed reduced activity in the dentate gyrus (DG) and subiculum (Sub) of db/db mice exposed to restraint stress. For all graphs, error bars represent the sem from (n=8) mice per genotype and asterisk (*) denotes statistical significance at $p < 0.05$ following bidirectional t-test.

Figure 3. Reductions in hippocampal BDNF prolong endocrine responses to stress in db/db mice. (A), Experiment schematic. Abbreviations: EPM, elevated plus maze; L/D, light/dark box. (B), Reporter expression (GFP) after bilateral injections of lentiviral vectors targeting the dorsal and ventral hippocampus. Scalebar=1mm. (C), Hippocampal BDNF protein levels are reduced in Wt mice injected with the knockdown vector (Wt-BDNFKD), relative to Wt mice injected with reporter construct (Wt-GFP). In db/db mice, stereotaxic injection of the overexpression vector (db/db-BDNFOE) normalized levels of hippocampal BDNF, relative to db/db mice injected with the reporter (db/db-GFP). (D), BDNF knockdown reduces open arm exploration in Wt mice (left) without affecting overall activity (right). In db/db mice, BDNF overexpression increased open arm exploration (left) without any effect on hypoactivity (right). (E), Wt-BDNFKD mice spent less time in the lit side of the arena, relative to Wt-GFP (left), while db/db-BDNFOE mice spent more time exploring the light side than db/db-GFP. Both groups of db/db mice crossed between the two sides less frequently than Wt mice (center). Analysis of the average duration of light side exploratory epochs (right) revealed that BDNFOE reduced anxiety in db/db mice and BDNFKD induced anxiety in Wt. (F), Lentiviral manipulation of hippocampal BDNF selectively regulates glucocorticoid recovery after 30min restraint stress without influencing basal corticosteroid levels. (Right), Comparison of the area under the curve (AUC) revealed increases in Wt-BDNFKD and reductions in db/db-BDNFOE, relative to genotype-matched controls that received the reporter construct. (G), Quantification of cFos+ nuclei in the PVH revealed increased activation after hippocampal BDNF knockdown in Wt mice and BDNF-dependent increases in db/db mice. (H), Peroxidase labeling for cFos in the PVH. Scalebar = 200 microns. For panel (C), bar height shows the mean of (n=4–6) mice per condition. For

panels (D-F), bar or symbol height represents the mean of (n=11–13) mice per condition. For panel (G), bar height represents the mean of (n=7) mice per condition. For all graphs, error bars represent the sem and asterisk (*) indicates statistical significance at $p<0.05$ following one-way ANOVA with Dunnet's post hoc.

Figure 4. Widespread disruption of stress-induced circuit activation reveals a pivotal role for hippocampal BDNF.

(A), Schematic depiction of the workflow for imaging, detection, and registration of tiled images. (B), Algorithmic detection of cFos+ cells in hippocampal and retrohippocampal regions revealed overlapping effects of diabetes and BDNF knockdown in the DG and ventral subiculum (SUBv). Micrographs (right) show representative images of cFos+ cells in the indicated conditions. Scalebar (left) = 200 microns. (C), ssTIF quantification of cFos induction in pallidal regions revealed BDNF-dependent reductions in the anterior bed nucleus of the stria terminalis (aBNST) and BDNF-independent reductions in the substantia innominata (SI). Micrographs (right) depict cFos labeling in the aBNST. (D), Positive correlation between manual cell counting and automated detection of cFos+ cells in the hippocampal dentate gyrus (left) and aBNST (right). (E), ssTIF analysis of cFos+ cells in cortical regions, as classified by the Allen Brain Atlas. (F), Parallel effects of leptin receptor deficiency and hippocampal BDNF knockdown on cFos induction in the basolateral (BLA) and basomedial amygdala (BMA). (G), Changes in hippocampal BDNF were necessary and sufficient for alterations in ventral striatal cFos induction after stress. (H), Reductions in hippocampal BDNF amplify stress-induced cFos induction in olfactory regions in db/db mice. (I), Venn diagram shows distinct and overlapping regional changes in cFos induction after stress, relative to Wt-GFP. For bar graphs, legend in (B) applies to panels (C, E-H). Bar height represents the mean of (n=7) mice per condition and error bars represent the s.e.m. Asterisks (*) indicate significant differences between Wt-GFP and db/db-GFP; crosses (†) indicate the effect of BDNFKD in Wt; and hashtags (#) denote effect of BDNFOE in db/db determined by ANOVA with FDR-corrected post hoc comparisons (Supplementary Table 2). For scatterplots (F), statistically significant correlations were determined at $p < 0.05$ using Pearson's correlation.

Figure 5. Hippocampal BDNF determines recruitment of anatomically connected networks after acute stress.

(A), Strategy for analysis of functional covariance. (B), Principal components analysis revealed that three groups of regions accounted for nearly half of the variability between groups (see Supplementary Table 3). (C), The absolute difference in cFos counts between paired brain regions was significantly increased in Wt mice injected with the BDNF knockdown virus (Wt-BDNFKD), relative to Wt mice injected with the reporter construct (Wt-GFP). db/db-GFP mice exhibited similar increases, which were eliminated by overexpressing hippocampal BDNF (db/db-BDNFOE). (D), Inter-regional correlations were reduced in Wt-BDNFKD and db/db-GFP mice, but not in db/db-BDNFOE mice. (E), Strategy for analysis of relationships between functional covariance and projection strength. (F), Distribution of analyzed regions across projection weights. (G), Regional co-activation is strongest in areas with greater anatomical connectivity. (H), Relationships between connectivity and clustering are degraded by loss of BDNF in db/db mice. For all graphs, asterisks (*) indicate significant difference ($p < 0.05$) between Wt-GFP and db/db-GFP; daggers (†) indicate the effect of BDNFKD in Wt; and hashtags (#) denote effect of BDNFOE in db/db as determined by one-way ANOVA (C), or two-way ANOVA (G-H) with Dunnet's post hoc. Error bars represent s.e.m. from ($n=7$) mice per condition.