Abnormal Recovery from Acute Stress in Huntingtons Disease Mice

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ABSTRACT

Introduction: Comparisons of hypothalamic dysfunction between Huntington's Disease (HD) patients and rodent models of HD have not always yielded similar results. Cortisol levels in HD patients have been contradictory, with reports ranging from hypo- to hypercorticoidism of morning measurements. Initial reports of major elevations in circulating corticosterone levels in the R6/2 mouse model of HD have only been followed up in one other closely related model, the R6/1 mouse, and the results were not perfectly congruent. To determine if abnormal stress hormones were a characteristic of disease, we examined diurnal and stress-induced corticosterone levels in multiple HD mouse models.

Methods: We analyzed serum corticosterone levels from R6/2, Q140 and BACHD mice either diurnally (R6/2) or after restraint stress and subsequent recovery (all 3 models). Glucocorticoid secretion was also examined (R6/2) following dexamethasone suppression and ACTH stimulation. Blood samples were collected either by submandibular puncture or as trunk blood at sacrifice. Plasma corticosterone levels were assayed by RIA. Regional brain glucocorticoid receptor (GR) levels were visualized using immunofluorescence.

Results: In the R6/2 mouse, basal levels of corticosterone, exhibited normal, sexually dimorphic diurnal rhythms but eventually became elevated towards end of life, after 13 weeks. The ability of the adrenal gland to produce corticosterone was not altered and the adrenal response to ACTH stimulation was only elevated in male R6/2 mice compared to littermates. Down regulation of GR receptors was only observed in the PVN at 16 weeks. R6/2 mice at both 6 and 12 weeks responded equivalently to littermates in the initial corticosterone response to restraint stress but serum corticosterone recovered from stress significantly more slowly. No differences were found in the initial stress response or in the recovery from stress for Q140 or BACHD mice, examined at 12 and 9 months, respectively.

Conclusions: We did not find evidence of major disruption in glucocorticoid signaling in HD mouse models. A significantly slowed recovery from stress observed in the R6/2 mouse suggests such disruptions are context dependent. These results suggest that disruption of the HPA axis was subtle and occurred only at advanced stages of disease represented by comparisons among these HD mouse models.

INTRODUCTION

Much attention has been paid to examining the multiple hormonal abnormalities associated with HD in both humans and mouse models in both the pituitary and peripheral tissues (van der Burg et al., 2009;Hult et al., 2010). Physiological stress can occur in HD as a correlate of many other disease-related changes, including loss of sleep (Lazar et al., 2015), disrupted circadian rhythm (Morton et al., 2005), autonomic function (Aziz et al., 2010) and metabolic changes (Gaba et al., 2005;Goodman et al., 2008). This array of homeostatic dysfunctions may be attributable to disruption of hypothalamic neurons and the hypothalamic-pituitary-adrenal (HPA) axis (Hult et al., 2010). Indeed, a number of laboratories report alterations in HPA function in affected individuals (Hult et al., 2010;Aziz et al., 2009;Leblhuber et al., 1995;Heuser et al., 1991;Saleh et al., 2009). Early morning serum cortisol levels were initially reported to be higher in male HD patients of unspecified disease stage (Leblhuber et al., 1995;Saleh et al., 2009). In contrast, more recent investigations did not detect significant changes in hormonal profiles between effected HD individuals and appropriate controls during a standardized day (Kalliolia et al., 2015). For morning cortisol levels, significant differences were not observed in peak, waking levels but for the calculated area-under-the-curve for increases above baseline over an hour of observation following waking in premotor, symptomatic, early stage individuals, compared to individuals after motor manifestation and family member controls (Hubers et al., 2015). One study has reported hypocortisolism among depressed early stage HD patients (Shirbin et al., 2013). Evening cortisol and ACTH levels were also reported either to be unchanged (Saleh et al., 2009) or to be higher in patients of both sexes over a range of HD stages (Heuser et al., 1991). Acute ACTH responses to cortisol releasing hormone (CRH) administration were lower in HD patients (Heuser et al., 1991).

Comparisons of hypothalamic dysfunction between HD patients and rodent models of HD have not always yielded similar results, suggesting methodological, contextual or disease progression related heterogeneities (van Wamelen et al., 2014). Variability in human genetic background is considerably greater than that of the mouse models. For example, the R6/2, Q140 and BACHD mice were initially developed on standard C57B6/CBA, C57BL/6, and FVB backgrounds, respectively (Mangiarini et al., 1996;Gray et al., 2008;Hickey et al., 2008). Yet despite their differences, these are well characterized, established genetic backgrounds for which many physiological parameters have been quantified (Jackson Laboratory, 2017). Such uniformity is not present in the human population. Human variability could contribute to the inability to uncover abnormal diurnal cortisol levels. To more fully understand how the genetic background and mouse model variability might affect the HPA axis in HD, we sought to examine the response to acute stress in a number of mouse models of HD. In this process, we not only addressed the immediate response to restraint stress, but we also examined diurnal corticosterone patterns and the ability to recover from stress.

METHODS

Mice: All animal husbandry and experimental procedures were approved by the University of MN Institutional Animal Use and Care Committee and comply with national and international standards. Mice were housed in groupings of two or four, kept on a 14:10 (0600h:2000h) light/dark cycle and given ad lib access to food and water. R6/2 mice were bred by crossing R6/2 males with C57B6/CBA F1 females. Two cohorts of R6/2 mice and their littermate controls were used. For initial studies, mice were obtained from UCLA (Dr. Michael Levine) and had polyglutamine repeats of 210-244. The second cohort was obtained from the CHDI

Foundation colony at Jackson Laboratories and had polyglutamine repeats of 120-142. Both cohorts were housed with nesting materials to reduce chronic stress (Van Loo et al., 2004). The second cohort was housed with nylabones and igloos as additional cage enrichment. Both cohorts developed clasping, became hypokinetic and disheveled and were sacrificed prior to becoming moribund. Heterozygous Q140 mice were also obtained from UCLA (Dr. Michael Levine) and were cross bred to produce homozygous offspring used in this study. Q140 repeat lengths ranged from 125 to 151. All BACHD mice (heterozygotes) were obtained from the Jackson Laboratory.

Blood Collection: Blood samples were collected by submandibular puncture (Golde et al., 2005) or as trunk blood at sacrifice without anesthesia. Approximately 100µl of blood was harvested in each submandibular sample. For diurnal measurements from any mouse, the AM sample (0700-1200h) and PM sample (1700-1900h) were separated by 2 weeks. Since the method and timing of blood collection can itself produce sufficient stress to elevate blood levels of corticosterone (Vahl et al., 2005), we took great care to not disturb a cage prior to blood collection and to obtain the sample within 2 min of moving or disturbing the cage. For all procedures, all mice from the same cage were handled simultaneously to avoid the stress associated with disruption of cage groupings (Gartner et al., 1980). Whole blood was allowed to clot before harvest of serum by centrifugation. Serum samples were stored at -20°C prior to assay.

Restraint Stress and Tissue Harvest: For restraint stress, cages of pair housed mice were moved into the experimental setting the evening prior to the experiment to avoid the stress associated with transporting the cages. Mice were restrained in their home cage within a 50 ml conical tube with an open tip for 30 min. After removal from restraint, a 100µl blood sample was obtained by submandibular puncture and the mouse was returned to the home cage to recover. After 60 min of recovery and for unstressed mice, trunk blood was collected by decapitation and brains and adrenals were harvested, weighed and frozen in isopentane on dry ice.

Corticosterone measurements: Serum samples were assayed in duplicate for corticosterone content using a commercial RIA kit (MP Biochemicals, LLC, Orangeburg, NY) as described previously (Ulrich-Lai et al., 2006). All samples were heated at 65°C for 30 min prior to assay to denature corticosterone binding globulin. The within-assay coefficient of variation of a standard sample across all RIA runs was 11.3% (Rodbard, 1974).

Dexamethasone Suppression and ACTH stimulation: In the *no stress* condition, mice were moved into the experimental setting on the evening prior to the experiment. In the *prior stress* condition, mice were placed in a novel cage in a new room the evening before and moved into the experimental room on the morning of the experiment. Following a blood draw, mice received a subcutaneous injection of 50 µg/100g body weight dexamethasone (Kier et al., 2005) at 0900h and were returned to their home cage for 6 hours. At 1500h, a blood sample was obtained and 1 µg/100g body weight ACTH (Kier et al., 2005) was injected intraperitoneally. Trunk blood was obtained 30 min later.

Blood Glucose: Serum glucose levels were determined with an Aviva Accuchek glucometer.

Immunocytochemistry, Image Processing & Quantification: Mice were sacrificed for tissue collection by pentobarbital overdose and transcardially perfused with saline and 4% paraformaldehyde. After decapitation, brains were dissected out and stored in 4% paraformaldehyde at 4ºC. Tissues from both R6/2 and control animals were sliced on a cryostat at 40µm thickness. Sectioned tissue was mounted on gel-coated slides and allowed to dry overnight. After several cycles of rinsing, tissue was placed in 0.5% Triton-X in PBS for 15

minutes followed by a blocking solution of 0.1% fish gelatin in PBS-0.5% Triton-X for 1 hour. Rabbit anti-mouse glucocorticoid receptor (GR, M-20 Santa Cruz biotechnology, Santa Cruz, CA) was diluted 1:400 in PBS, 3% goat serum, 0.2% fish gelatin and 0.5% Triton-X. Tissues were incubated in primary antibody for 24 hours at 4ºC followed by rinsing. Subsequently, brain sections were incubated overnight at 4º C in secondary antibody, DyLight 488 goat anti rabbit IgG (Jackson ImmunResearch, West Grove, PA) diluted 1:400 in PBS, 3% goat serum, 0.2% fish gelatin and 0.5% TX-100. For nuclear counterstain, 500 nM DAPI (Invitrogen Molecular Probes, Eugene, OR) was added to brain slices for 5 minutes. After rinsing, tissues were dehydrated in alcohol and cover-slipped with DPX.

All images were collected on an Olympus FluoView 1000 confocal microscope (Olympus America Inc, Center Valley, PA) at wavelengths appropriate for secondary antibody and the DAPI counterstain using a 40x oil immersion objective at identical optical settings. Quantification of the 488nm signal from tissues stained on the same day was performed in Olympus Fluoview 2.0. Regions were created over neuronal nuclei using the DAPI image and the average intensity per pixel was computed for comparison across genotypes. For all anatomical areas of interest, a pair of images were captured, one of DAPI nuclear stain, and another of FITC anti-GR antibody stain.

Quantification of fluorescence was processed in capture-Scope/Metamorph (Molecular Devices, Sunnyvale, CA). DAPI counter stain was used to create regions of interest around the nucleus of visualized tissue. This mask with regions of interest was then transferred to the corresponding FITC image captured at the same region and depth level. The software then logged the intensity profile within the nuclear region for the anti-GR antibody.

Statistics: All statistical comparisons were performed in GraphPad Prism 6.01 (San Diego, CA).

RESULTS

Basal Serum Corticosterone

To assess the response to stress, the most severe mouse model of HD, the R6/2 mouse with over 200 polyglutamine repeats, was first considered. Corticosterone levels in serum sampled from non-stressed R6/2 and littermate mice exhibited normal diurnal and sexually dimorphic variation (Fig. 1A). As expected, when comparing sex and time of day within wild type mice by two-way ANOVA, female levels exceeded male levels ($F(1,53)=8.633$, $p=0.0049$). This sex difference was seen in R6/2 mice as well ($F(1,48)=7.756$, $p=0.0076$). However, the increased variability of R6/2 measurements prevented the male/female differences from reaching significance in post-test comparisons of the AM measurements. Thus, in all further studies, corticosterone values were not combined across gender; statistical measures were applied in single sex comparisons.

Morning corticosterone levels were lower than evening samples in both genotypes. These patterns held for the R6/2 mice as well as their wild type littermates, provided the R6/2 mice did not exceed 13 weeks of age (Fig. 1A). When the effects of genotype and time of day were analyzed among males or females in two-way ANOVAs, variation was attributable only to the diurnal rhythm and not genotype (statistics in figure legend). In all analyses, the variability of the R6/2 measurements exceeded that of their littermates. For most of their lifespan, R6/2 mice did not appear to have higher basal corticosterone levels than littermates or altered diurnal rhythms.

The lifespan of R6/2 mice is quite limited. Lifespan in colonies has been reported to vary between 12 and 20 weeks (Mangiarini et al., 1996;Carter et al., 2000). In this cohort, the mean time to moribund status was approximately 13 weeks. In those male mice that survived past 13 weeks, basal corticosterone levels were markedly elevated and lost any semblance of a diurnal rhythm (Fig 1B). For females, morning levels appeared higher but did not reach significance for this limited sample. Sample size also precluded evening testing. Thus major disruption of basal corticosterone regulation appeared to characterize end stage disease.

Analysis of serum glucose concentration in this initial cohort of mice revealed that they were diabetic after approximately 10 weeks of age (184.6 ± 64.5 mg/dL, n=33 for 10-13 week old littermates, 304.2 ± 97.4 mg/dL, n=19 for similarly aged R6/2 mice, $p<0.001$, two-tailed t test). The presence of elevated serum glucose did not alter the normal diurnal corticosterone variations. To prevent this endocrine disorder from complicating further analysis, we switched to R6/2 mice from the re-derived Jackson colony, which were not diabetic (162.9 ± 17.5 mg/dL, n=16 for littermates, 188.7 ± 77.9 mg/dL, n=14 for R6/2, all 10-12 week of age). This non-diabetic cohort was used in all subsequent experiments.

Stress Responses

Previous experiments in R6/1 and R6/2 HD mice suggested that the hormonal response to stress might be altered (Du et al., 2012;Dufour and McBride, 2016). To test this idea, R6/2 mice were subjected to 30 min of restraint stress followed by 60 min of recovery. For the restraint stress experiments, initial values at 0 min were obtained from separate mice not exposed to stress, so that values at 30 min represent the response to restraint alone and not the stress of blood loss. At both 6 and 12 weeks of age, no differences were observed in the corticosterone levels produced by restraint stress (Fig. 2, 30 min). However, after 60 min of recovery in the home cage, corticosterone levels in R6/2 mice remained or trended elevated compared to that of littermates (Fig. 2, 90 min).

The presence of this slowed recovery from stress in females at early stages of R6/2 disease progression suggested that prolonged stress responses might characterize early stage disease. To explore this idea, similar restraint stress responses were obtained from milder mouse models of HD, Q140 and BACHD mice at 12 months and 9 months respectively (Fig. 3). No genotype specific changes in the response to stress or its recovery were observed in either of these mouse models. For a limited number of BACHD animals tested at 12 months, a similar pattern was seen with no genotype-specific differences in corticosterone values after restraint or recovery.

The slowed recovery after stress in the R6/2 suggested disruption of the feedback pathway regulating the HPA axis. Circulating corticosterone normally acts on glucocorticoid receptors (GR) in the hippocampus, hypothalamic paraventricular nucleus (PVN) and pituitary to limit its own production. Activation of hippocampal GR sends inhibitory feedback to hypothalamic nuclei secreting corticotrophin releasing factor (CRF), resulting downstream in decreased corticosterone production and release from the adrenal (Laryea et al., 2015b). To determine if this pathway was active, dexamethasone, a synthetic glucocorticoid, was administered to activate GR receptors and generate decreased corticosterone levels. In male R6/2 mice and littermates, dexamethasone administered to unstressed mice at 0900h, prevented the expected diurnal rise in corticosterone levels 6 hours later (Fig. 4B). Some female R6/2 mice escaped dexamethasone suppression and exhibited a diurnal corticosterone increase (Fig. 4A). If the male mice were subjected to mild stress prior to dexamethasone administration,

corticosterone levels in littermates, but not R6/2 mice, were completely suppressed 6 hours later (Fig. 4C). In this experiment, the mild stress consisted of overnight housing in a novel cage in a novel room with transfer to the experimental room immediately prior to obtaining the blood sample. Dexamethasone was able to decrease corticosterone levels in the R6/2 males, but not completely, reminiscent of the partial recovery seen after restraint stress.

To determine the adrenal sensitivity to ACTH stimulation, ACTH was injected into the mice after 6 hours of dexamethasone suppression (Fig. 4D). In both male R6/2 and littermates, adrenals responded to ACTH with a large surge in serum corticosterone. Corticosterone levels after ACTH injection were significantly elevated in male R6/2 compared to littermates ($p=0.038$, two tailed *t* tests) and trended higher in females ($p=0.053$). This would be expected if the adrenal cortices in the R6/2 mice were larger than littermates and able to produce more corticosterone (Table 1). Indeed, at sacrifice, the corticosterone content of the adrenals from these mice was proportional to serum corticosterone levels (Fig. 5), indicating normal function in adrenals with possibly hyperplasic cortices. The consistent relationship between adrenal content and secreted corticosterone suggests an absence of abnormalities in R6/2 for corticosterone distribution, binding and metabolism.

Additionally, glucocorticoid receptors (GR) were visualized with immunohistochemistry in various parts of the mouse brain to determine if these hormone feedback pathways might be altered regionally. To examine the integrity of the central feedback pathway, glucocorticoid receptors (GR) were identified by immunocytochemistry in brains of R6/2 and littermate mice. The immunocytochemistry was performed on the diabetic cohort of mice. The data is presented because it provides information on disease progression. At 8 weeks, GR levels in the hippocampus, paraventricular nucleus, and the prefrontal cortex (PFC) of R6/2 mice were not different from those of littermates (Figs. 6 and 7). At 16 week of age, GR levels in the PVN were decreased in R6/2 mice but were unchanged in other areas of the brain.

DISCUSSION

These studies examined glucocorticoid function in the HPA axis of the R6/2 mouse model of HD. In this rapidly progressive disease model, basal levels of the murine stress hormone, corticosterone, exhibited normal, sexually dimorphic diurnal rhythms but eventually became elevated towards end of life, after 13 weeks. These observations were independent of the CAG repeat length (data not shown) or the diabetic status of the mice. The ability of the adrenal gland to produce corticosterone was not altered and the adrenal response to ACTH stimulation was only elevated in male R6/2 mice compared to littermates. Down regulation of GR receptors was only observed in the PVN at 16 weeks. R6/2 mice at both 6 and 12 weeks responded equivalently to littermates to restraint stress but recovered significantly more slowly. While more slowly progressing, the Q140 mice at 12 mo and BACHD mice at 9 mo were tested at ages corresponding to the onset of symptoms in these models (Gray et al., 2008;Hickey et al., 2008;Menalled et al., 2009). However, both Q140 and BACHD mice responded to and recovered from restraint stress in a manner equivalent to littermates. These results suggest that disruption of the HPA axis was subtle, context dependent and occurred only at advanced stages of disease represented by comparisons among these HD mouse models.

Previously, a specific HPA axis abnormality was reported in females, but not male R6/1 mice at 12 week (Du et al., 2012). Not only were females' corticosterone levels increasingly elevated over the hour following a 10 min forced swim test, but an ACTH challenge led to exceptionally high serum corticosterone (Du et al., 2012). We did not detect such female-

specific abnormal HPA responses with the R6/2 mice. While female corticosterone levels generally exceeded those of males, the diurnal pattern, response to restraint stress and recovery from stress followed similar patterns in both males and females. For the sexually divergent results reported here, the absence of statistical significance in some instances for either sex was most likely attributable to the low number of mice involved. This applies to male R6/2 mice at 6 weeks that trended towards elevated recovery from stress (Fig. 2C) and the highly elevated but variable response to ACTH in females (Fig. 4D).

In previous studies reporting progressive increases in basal stress levels in R6/2 mice (Bjorkqvist et al., 2006), samples were acquired following anesthesia, in itself a known stressor (Vahl et al., 2005). We were surprised that we were unable to replicate these observations reporting progressively elevated afternoon corticosterone levels among R6/2 mice from 6 week of age and older. Since anesthesia and the handling to inject anesthesia would be expected to cause stress, this could contribute to the responses they reported (Vahl et al., 2005). In the current work, we were careful not to disturb the mice in any way prior to obtaining the blood sample used for basal corticosterone assessment. In instances where we tried to obtain a sample from a third or fourth mouse from a cage or if activity or traffic in the animal room had disturbed the colony, elevated corticosterone levels were often encountered (unpublished observations). Thus we limited our collections of the data reported here to pair housed mice from previously undisturbed cages. Our inability to replicate the longitudinal increase in plasma corticosterone levels calls into question whether glucocorticoid abnormalities contribute substantially to disease progression.

Elevated daytime basal corticosterone levels were observed in a previous study of R6/2 mice in which blood was sampled every four hours (Dufour and McBride, 2016). This elevation did not appear until mice were 10 weeks of age (Dufour and McBride, 2016), similar to our results after 13 weeks. In early stage, medication free HD patients, diurnal measures of plasma cortisol revealed elevations in late morning, early afternoon and during sleep but not of the apogee or nadir values resulting in an overall subtle increase in total daily cortisol secretion (Aziz et al., 2009). This pattern is reminiscent of what we report here in the R6/2 mice. The daily early morning and evening corticosterone levels are not different from non-diseased mice but after stress-induced elevations, corticosterone was slow to return to baseline. One could extrapolate this result to reinterpret the daytime human data as reflecting a failure to return to baseline after perturbations associated with daily activities, either through a reduced clearance mechanism or continued release.

Surprisingly, the response to the restraint stress itself did not produce a greater response in any of the HD mice compared to their littermate controls. Rather the recovery from stress was prolonged only in the more severe R6/2 model at early and later disease stages and not in the Q140 or BACHD models. Previously, male, but not female, R6/2 mice were observed to have a prolonged recovery from restraint stress in a paradigm where blood was drawn at 30 min intervals from each mouse over two hours (Dufour and McBride, 2016). With these repeated blood draws, a higher initial corticosterone level and a subsequently blunted relative increase at the end of the restraint were observed (Dufour and McBride, 2016). The contrasts between these two studies may be attributable to the differences in sampling frequencies. While the longitudinal design may have greater experimental validity, stress added from repeated samplings could confound the results. In neither case, however, were the immediate responses to stress elevated.

The failure to recover from stress is often attributed to inadequate feedback activation of hippocampal GR receptors (see below). However, it could also serve a homeostatic function. As a synthetic glucocorticoid, dexamethasone has been used to investigate downstream effects of chronic GR activation in HD mice and drosophila (Maheshwari et al., 2014;Diamond et al., 2000). In these models, daily dexamethasone injections increase expression and activation of the transcription factor HSF1 which in turn stimulates a protective heat shock response, decreasing aggregate loads and ameliorating phenotypes (Maheshwari et al., 2014). The maintained elevation of corticosterone reported in these R6/2 restraint studies, may be a way to similarly activate HSF1 to mount a homeostatic, protective response following stress.

We encountered variability in response to dexamethasone suppression among the limited number of mice available for our studies. Unstressed male mice responded appropriately, suppressing diurnal corticosterone increases. In mildly stressed male mice, dexamethasone partially suppressed corticosterone responses. Female R6/2 mice inconsistently suppressed diurnal corticosterone increases. The lack of complete dexamethasone suppression in some of the R6/2 mice may reflect the same mechanisms responsible for their inability to recover rapidly from restraint. An absence of a response to dexamethasone, termed dexamethasone non-suppression, may be common. Dexamethasone non-suppression is age dependent and characteristic of ~38% of a population of unselected, hospitalized psychiatric patients who received a dexamethasone suppression test (Keitner et al., 1992). Among depression sufferers, dexamethasone non-suppressors have been linked to a greater risk of suicide (Lester, 1992;Mann et al., 2006). In HD gene carriers, depressive symptoms or suicidality were not associated with HPA axis activity (Hubers et al., 2015). However, this study did not report the presence of dexamethasone non-suppressors in their cohort which had 'mild suicidality scores' (Hubers et al., 2015).

Both the slowed recovery after stress and a variable response to dexamethasone, might be indications that the feedback regulation of the stress response is impaired. Yet, we only observed a steady state decrease in GRs in the PVN late in R6/2 lifespan and did not observe decreases in GR receptor density in hippocampus, cortex or striatum at late stage disease. This change could not account for the slowed recovery after stress beginning at 6 weeks. Following restraint stress, the expression level of GR receptors varies on an hourly and region specific basis, with prominent decreases occurring one hour after stress in the PFC, but not hypothalamus or hippocampus (Gadek-Michalska et al., 2013). With repeated stress, this decrease in GR expression reverses to a potentiation (Gadek-Michalska et al., 2013). Our data did not have this temporal resolution, reflecting only the steady state level of GR expression at sacrifice.

The role GR changes play in R6/2 disease progression is not obvious. The absence of a decrease in GR until 16 week in the R6/2 brain is consistent with the observed regularity of diurnal corticosterone release as the PVN is known to control the release of CRH (Laryea et al., 2015b). The PVN is under the control of both the suprachiasmatic nucleus and peripheral adrenal clocks (Laryea et al., 2015b). While the GR data acquired from diabetic R6/2 brains may not reflect the physiological state of the non-diabetic mice, they do suggest that GR levels remain normal in both hippocampus and hypothalamus during early disease. The loss of GR levels in PVN late in disease may reflect severe disease or the onset of the agonal state. When co-expressed with mutant huntingtin (muhtt) in vitro, GRs appear to decrease the nuclear accumulation of muhtt aggregates and promote diffuse cytoplasmic muhtt localization (Diamond et al., 2000). Thus the observed decrease in PVN GRs could promote muhtt aggregation in a disease-stage specific manner. How these shifts in GR might influence disease progression

depends upon context and whether aggregates are considered to be toxic or protective (van Hagen M. et al., 2017;Arrasate and Finkbeiner, 2012). Given the minimal alterations in GR levels in the diabetic R6/2 brains, it may be reasonable to assume a similar absence of GR dysregulation in the non-diabetic R6/2 brains. However, this remains to be verified in future studies.

Genetic suppression of GR receptor in the PVN over a lifetime (Sim1Cre-GRe3Δ) resulted in elevated basal corticosterone secretion followed by elevated restraint stress corticosterone responses in male mice by early adolescence and in female mice by adulthood (Laryea et al., 2015a). Notably though, these mice retained diurnal rhythmicity of corticosterone secretion (Laryea et al., 2015a). Additionally, these mice also displayed an elevated adrenal response to ACTH (Laryea et al., 2015a). While similar in this latter aspect, the corticosterone secretory pattern observed in R6/2 mice did not reveal increased responses to stress and is therefore inconsistent with an early loss of GR in the PVN.

The corticosterone phenotype of the R6/2 mice does resemble that of mice with a conditional knock out of the GR receptor in the pituitary ($GR^{POMCCre}$) (Schmidt et al., 2009). In adult $GR^{POMCCre}$ mice, after stress, corticosterone levels return to normal more slowly and dexamethasone suppression is also reduced. These appear to be a consequence of a transient increase in GR activation early in development that presumably reprogrammed the HPA axis for hyper-reactivity to stress later in life (Schmidt et al., 2009). Whether or not such a stress occurs in HD mice during P1-P12, the stress hypo-responsive period, remains to be explored.

This study was underpowered from the beginning as consistent large numbers of mice were not available for each set of measurements. However, the data reported here suggests that disturbances in glucocorticoid signaling in HD are subtle and may be more associated with the recovery from stress than from any chronic change in physiology. To fully examine regulation of the HPA axis and stress responses in HD mice, careful examination of both GR and the closely related mineralocorticoid receptor expression and activity would be required during stress and the recovery after stress.

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Table 1. Adrenal and Body Weights of unrestrained mice younger than 13 weeks of age. Data are means with standard deviations in parentheses. * $p<0.05$ comparing R6/2 to LM of same sex, two tailed *t* test.

	LM Females	LM males	R6/2 females	R6/2 males

Adrenal Weight (mg)	6.23 (0.84)	5.84 (2.75)	8.90 (2.54)	7.50 (1.16)
Body Weight (g)	20.2 (1.5)	26.2 (3.3)	18.6 (3.6)	25.0 (3.0)
Ratio (mg/g)	0.309 (0.034)	0.222 (0.102)	0.483 (0.120)	0.304 (0.061) *
N	3	10	8	5

FIGURE LEGENDS

Fig. 1 Diurnal variation in basal serum corticosterone levels in R6/2 mice (red) and littermate controls (black). A) Morning and evening corticosterone levels did not differ between R6/2 mice and controls for mice up through 13 weeks of age (range 5-13 weeks). Diurnal variation, but not genotype, accounted for a significant amount of the variability in 2 way ANOVAs comparing time of day and genotype for each sex (time of day: $F(1,41)=4.23$, $p=0.046$ for females; $F(1,60)=16.46$, $p=0.0001$ for males). B) Above 13 weeks of age, surviving male R6/2 mice lost the diurnal corticosterone rhythm and levels rose significantly over controls in which diurnal rhythms persisted (2 way ANOVA, for time of day $F(1,19)=0.4704$, $p=0.5011$; for genotype $F(1,19)=12.53$, $p=0.002$). For females, the morning levels trended upward. Fewer females survived to this age so evening measurements were not taken. Note the difference in ordinate scales between A and B. Data represent separately aggregated male and female values. *** $p<0.001$, comparing R6/2 to littermates, Bonferonni post-test after two way ANOVA. Bars represent means ± sem. N = 6-21 in A, 3-8 in B.

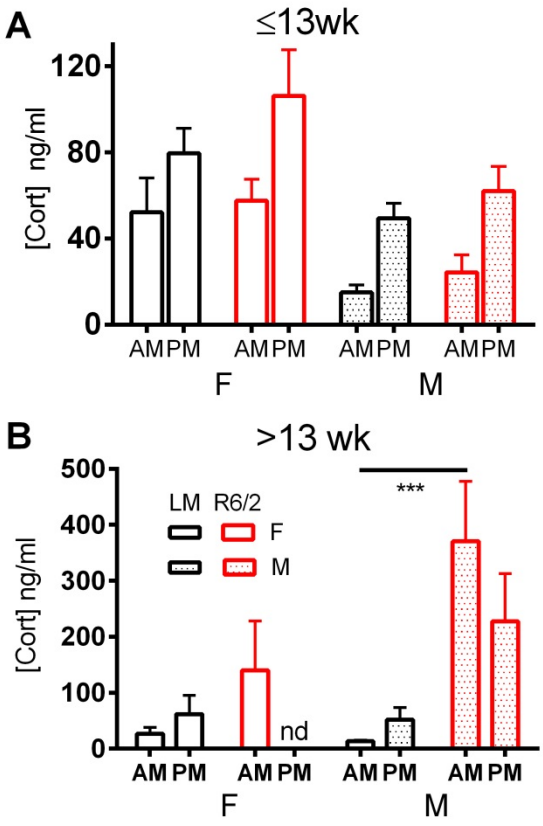


Fig. 2. Both female and male R6/2 mice recover more slowly from restraint stress compared to littermate controls. Corticosterone levels were obtained at the end of 30 min of restraint stress and following 60 min of recovery from stress in the home cage for 6 week (A, C) and 12 week old (B, D) littermate (black) and R6/2 mice (red). Since corticosterone levels were expected to decrease during recovery from stress and would provide significant variability if compared to the levels immediately after stress, two-tailed *t* tests were applied at single time points. At both ages, corticosterone levels only differed across genotypes at the end of the recovery period. * $p<0.05$, ** $p<0.01$. Compared to littermates, for R6/2 male mice at 6 week (C), $p=0.057$ for 90 min. Mean ± sem, N=8-9 (A), 2-4 (B), 2-5 (C, D).

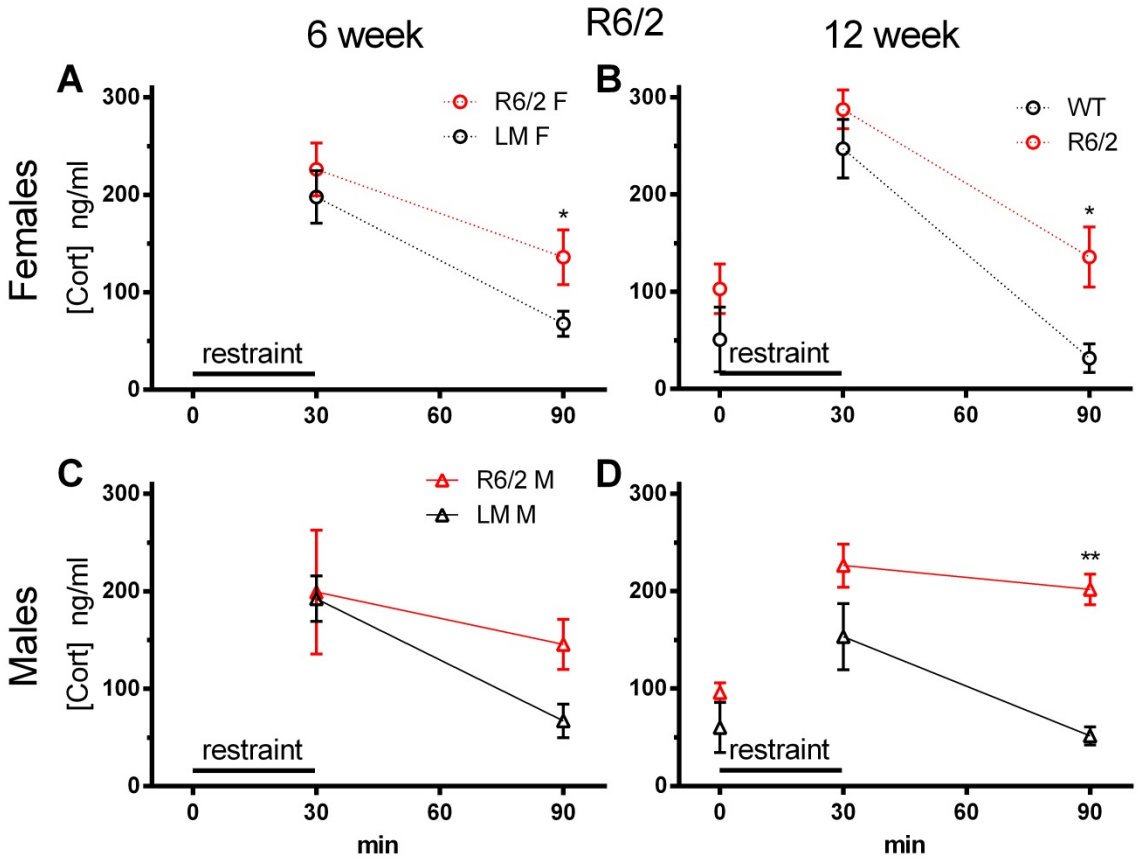
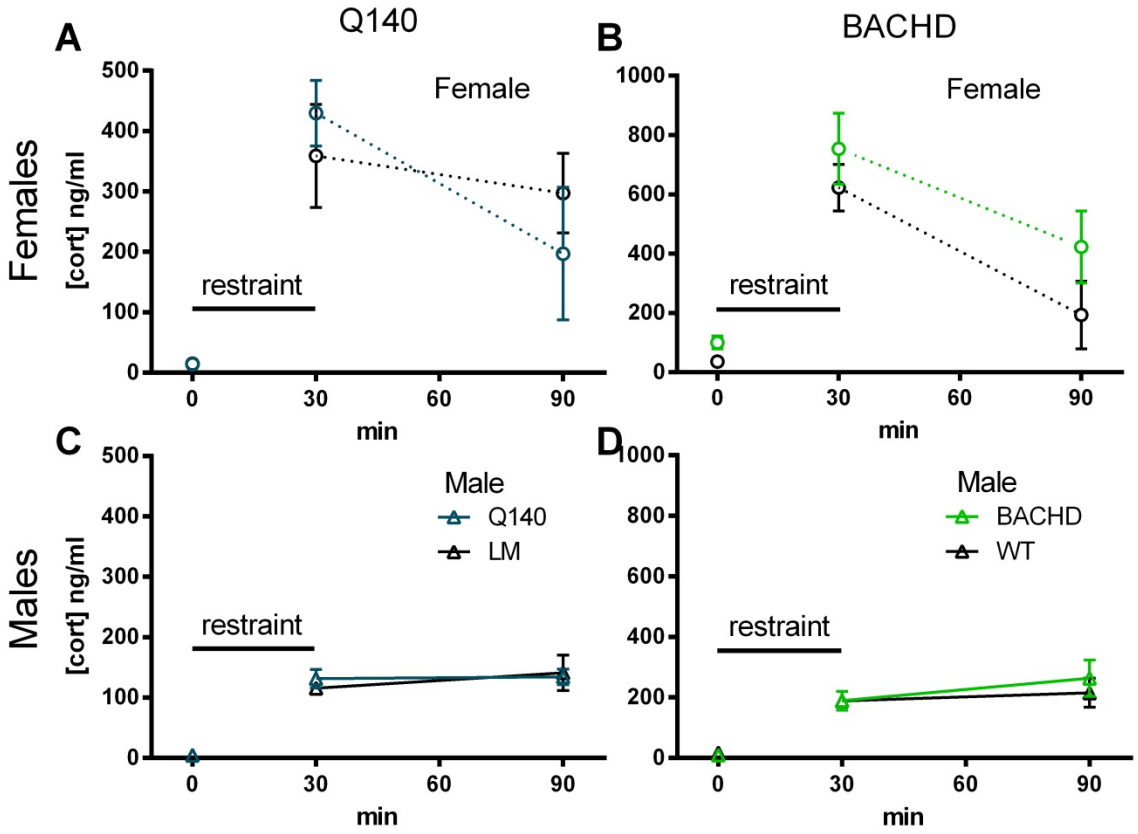


Fig. 3. Response to restraint stress in the Q140 mouse at 12 mo (A,C) and the BACHD mouse at 9 mo (B, D). Corticosterone levels were obtained at the end of 30 min of restraint stress and following 60 min of recovery in the home cage. Neither Q140 (blue) nor BACHD (green) mice exhibited abnormal stress responses or recoveries compared to control mice (littermates for Q140s, wild type for BACHD, black; two tailed *t* tests all n.s.). Mean ± sem, N = 2-8 (A), 5-7 (B), 5-13 (C), 5-9 (D).



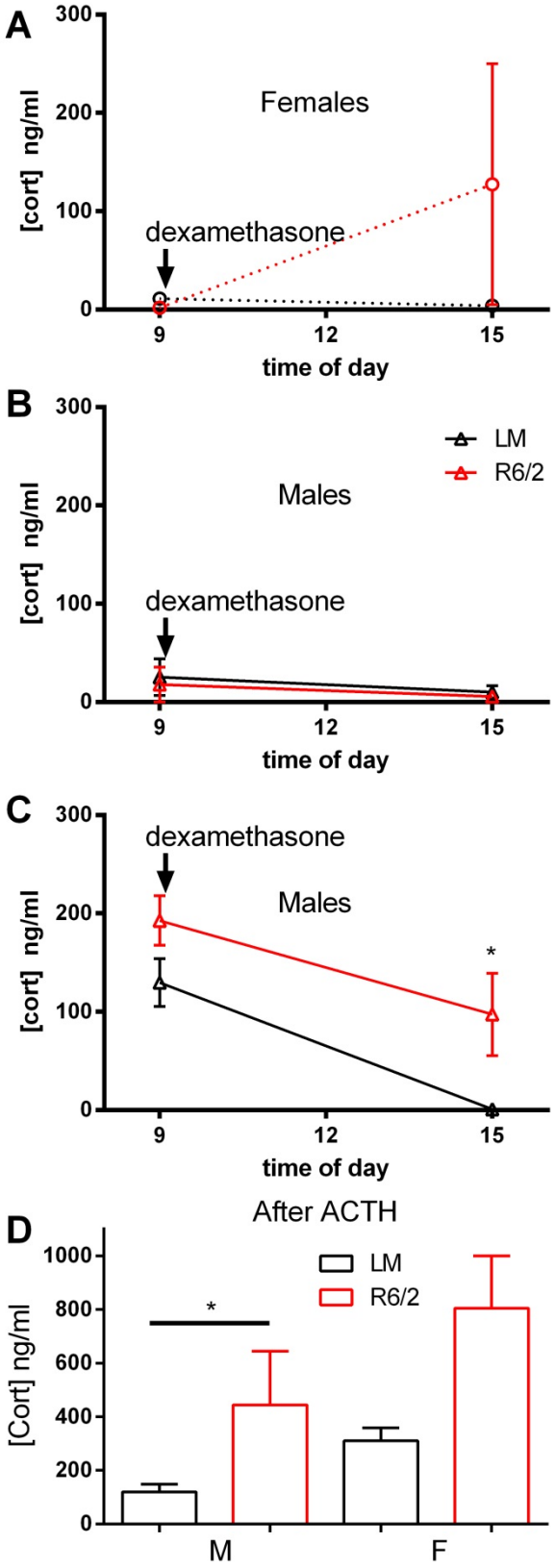


Fig. 4. Corticosterone levels in response to dexamethasone suppression and ACTH stimulation in R6/2 mice and littermate controls at 10 weeks. (A, B) Dexamethasone (50µg/100g body weight) administration completely suppressed the diurnal rise in corticosterone levels in unstimulated male R6/2 (red) and both male and female littermates (black) in the absence of any prior stress. Inconsistent results in R6/2 females increased variability. (C) In male mice exposed to mild overnight stress, dexamethasone only produced a partial suppression. Variation was attributable to genotype ($F(1,24)=18.41$, $p=0.0003$, 35%) and dexamethasone treatment ($F(1,24)=9.31$, $p<0.0055$, 17%) but not interaction (two way ANOVA). Corticosterone levels in R6/2 mice differed significantly from littermates 6 hr after dexamethasone treatment, $p=0.042$, Bonferroni post-test. (D) Injection of ACTH in the dexamethasone treated mice produced an increase in serum corticosterone. * $p<0.05$, two tailed *t* test. Data points are mean ± sem, N=2-3 (A), 2-6 (B), 4-10 (C), 2-5 (D).

Fig. 5. Adrenal and serum corticosterone levels increased in parallel in both R6/2 (red) and littermate control (black) mice. The corticosterone levels found in serum at sacrifice are plotted against the amount of corticosterone per mg of harvested adrenal tissue. Line represents a linear regression on all 27 data points whose slope was significantly different from zero ($p<0.001$).

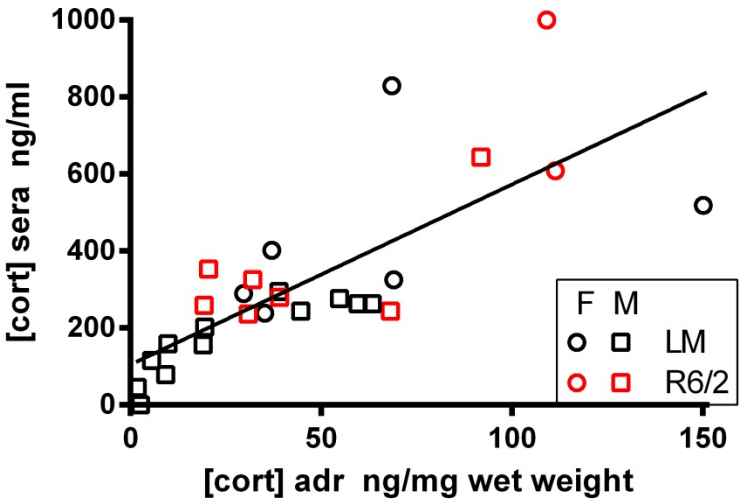


Fig. 6. Glucocorticoid receptor immunostaining in nuclei from hippocampus (HPC), prefrontal cortex (PFC), paraventricular nucleus (PVN) and striatum (STR) from littermates (wt, left column) and R6/2 (right column) mice.

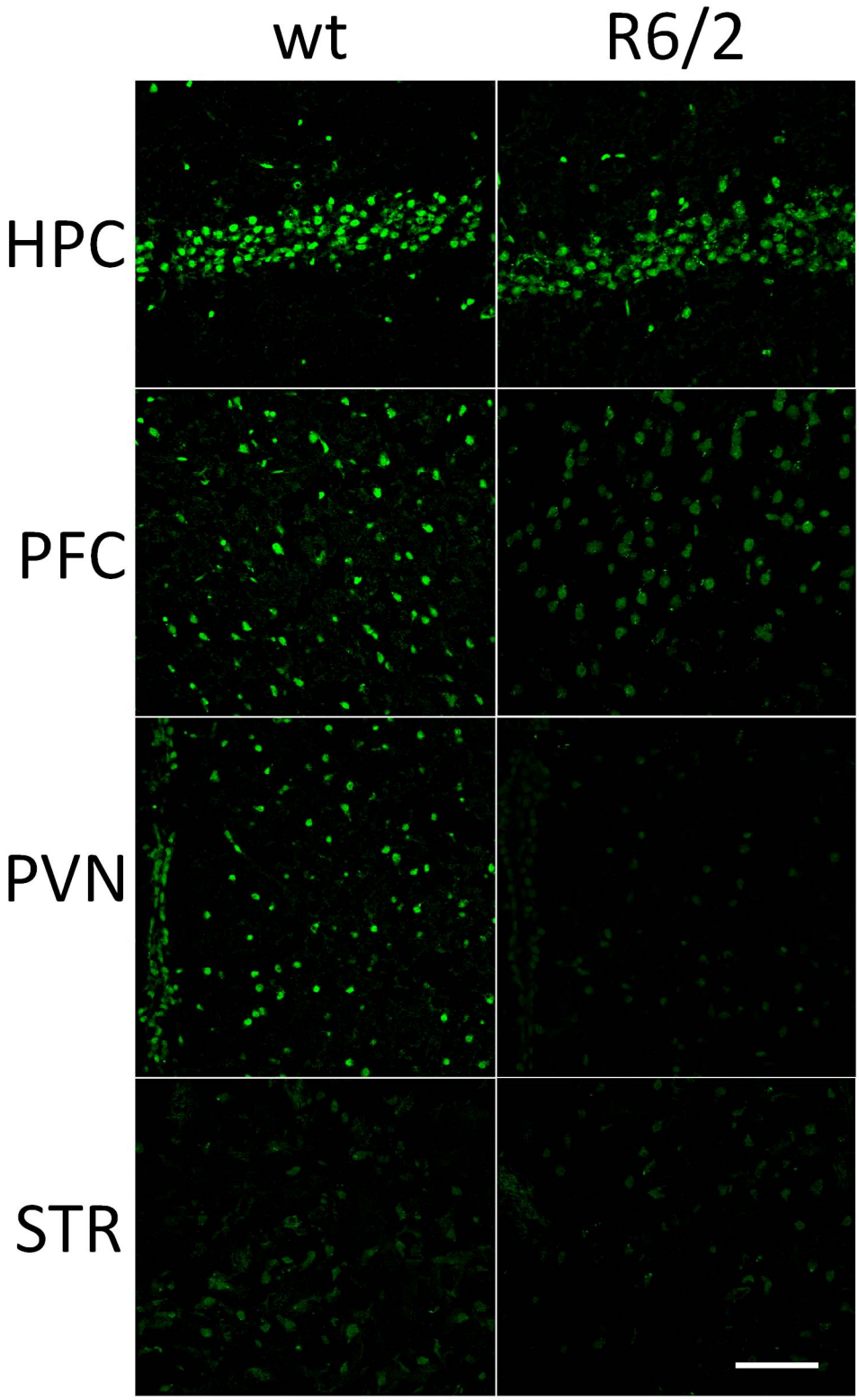
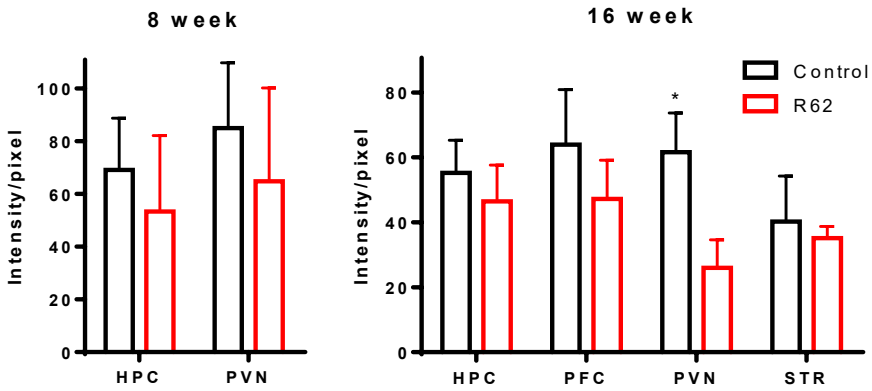


Fig. 7. Quantification of immunocytochemistry for GR in indicated regions of R6/2 and littermates brains at 8 and 16 weeks of age. HPC, hippocampus; PVN, paraventricular nucleus; PFC, prefrontal cortex; STR, striatum. Mean ± sem, N=3-6.



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