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The Thyroid Hormone-Regulated Corepressor Hairless Associates with Histone Deacetylases in Neonatal Rat Brain

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Thyroid hormone (TH) influences multiple aspects of neural development, presumably by controlling the transcriptional activity of TH receptors to modulate gene expression. The mammalian *hairless* (*hr*) gene is likely an important component of TH action as 1) *hr* expression is directly regulated by TH in brain, and 2) the protein encoded by *hr* (Hr) acts as a corepressor, facilitating transcriptional repression by unliganded TH receptors. Here we examine the properties of endogenous Hr in developing rat brain. Using coimmunoprecipitation, we show that Hr interacts with TH receptor and histone deacetylases (HDACs) in brain extracts. We find that inhibition of HDAC activity impairs Hr-mediated transcriptional repression, indicating that Hr-HDAC interaction is functionally significant. To identify potential sites of Hr action in developing brain, we assessed *hr* transcript and protein expression. We show that *hr* is broadly expressed in brain and overlaps with the expression of multiple HDACs in multiple regions including cortex, hippocampus, and cerebellum. Additionally, Hr expression is TH sensitive and developmentally regulated. The striking correlation of Hr expression with brain regions, cell types, and developmental stages influenced by TH, together with its function as a corepressor, suggests Hr is a key mediator of TH action in developing brain. (*Molecular Endocrinology* 16: 2547–2560, 2002)

COORDINATED CHANGES IN gene expression underlie many developmental and physiological processes. A subset of changes in gene expression in the developing mammalian brain are under the control of thyroid hormone (TH). In the rat brain, TH influences developmental processes in multiple brain regions during a critical developmental period (first three postnatal weeks) (1). Specific changes caused by neonatal TH deficiency include altered dendritic structure of hippocampal and cortical pyramidal cells and cerebellar Purkinje cells (reviewed in Refs. 1 and 2). In hypothyroid cerebellum, migration and differentiation of granule cells are delayed (3, 4). Myelination is reduced throughout the brain, due, in part, to delayed oligodendrocyte differentiation (5, 6). The morphological defects caused by neonatal TH deficiency likely underlie severe neurological disorders such as mental retardation, deafness, and ataxia suffered by humans with low levels of TH during development (7–9).

TH-regulated gene expression is mediated through nuclear receptors that either activate or repress the transcription of specific genes in response to ligand binding (10). Specific genes, the expression of which in the brain is TH dependent, make up the genetic program set in motion by TH; this program is thought to mediate the multiple and diverse effects of TH on brain development. The finding that the mammalian hairless (*hr*) gene is a direct target of TH in the developing brain suggests it is a component of this program and therefore plays a role in neural development (2, 11, 12). *hr* was originally identified in mice that exhibit congenital hair loss and was subsequently identified in humans that show an analogous phenotype (13, 14). The importance of *hr* in brain is supported by evidence that *hr* mutant mice exhibit neurological phenotypes that include altered neuronal morphology, inner ear defects, and abnormal retinal cytoarchitecture (15, 16).

We have shown that the protein encoded by the *hr* gene (Hr) functions as a nuclear receptor corepressor (12, 17). As a corepressor, Hr interacts with and enhances transcriptional repression by unliganded TH receptors (TRs). Like other nuclear receptor corepressors [silencing mediator of retinoid and thyroid hormone receptor (SMRT) and nuclear receptor corepressor (N-CoR)], Hr binds to TR via two independent domains and contains multiple, separable repression domains (Fig. 1A) (17–21). In addition, Hr associates with histone deacetylases (HDACs), suggesting that Hr mediates repression through HDAC activity (17).

To determine whether Hr acts as a corepressor in brain, we have examined the properties of endogenous Hr. We show that under physiological conditions Hr associates with TR and HDACs, components of the

Abbreviations: CoIP, Coimmunoprecipitation; COUP-TF, chicken ovalbumin upstream promoter transcription factor; HDAC, histone deacetylase; *hr*, hairless gene; Hr, protein encoded by *hr*; MBP, myelin basic protein; MLV, Moloney murine leukemia virus; N-CoR, nuclear receptor corepressor; p0, p13, p21, postnatal d 0, d 13, and d 21; Pcp-2, Purkinje cell protein 2; SMRT, silencing mediator of retinoid and thyroid hormone receptor; TH, thyroid hormone; TR, TH receptor; tk-luc, thymidine kinase luciferase; TRE, TH response element; TSA, trichostatin A.

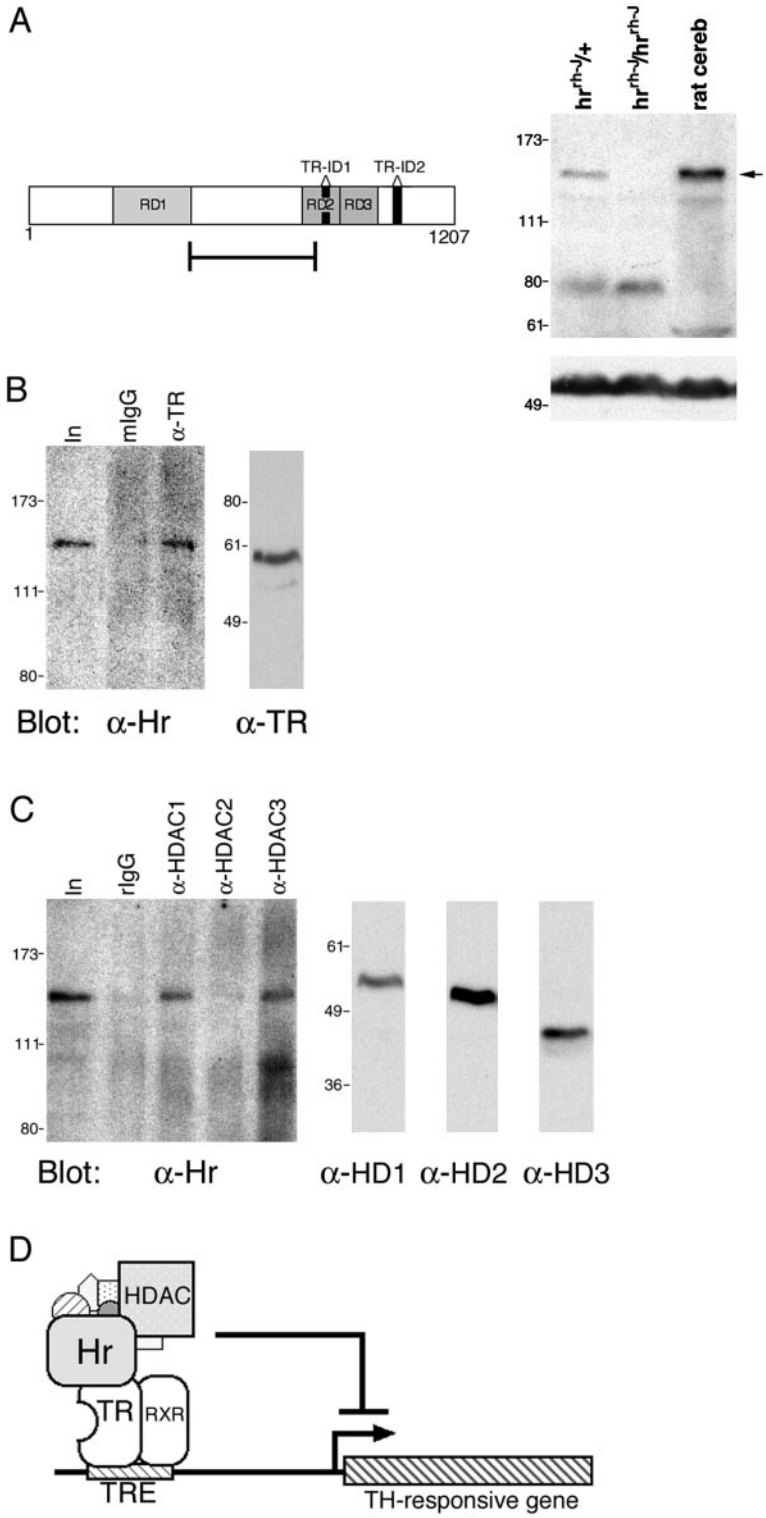

Fig. 1. Hr Associates with TR and HDACs in Rat Brain

A (*left*), Schematic representation of rat Hr with repression domains (RD) and TR interacting domains (TR-ID) *highlighted. Bracketed line* indicates region of Hr used to generate Hr-specific antiserum (amino acids 450–730). A (*right*), Hr-specific antiserum detects endogenous Hr protein. *Top panel*, Western analysis with Hr antiserum on cerebellar extracts prepared from mouse and rat. $hr^{rh\text{-}J}$ mice carry a mutation in the *hr* gene that results in more than 95% reduction of *hr* mRNA. *Arrow* indicates a band migrating at the predicted size of Hr (~135 kDa) detected in cerebellar extracts prepared from p28 rat and heterozygous *hr* mice ($hr^{rh\text{-}J}/+$) but not homozygous *hr* mice ($hr^{rh\text{-}J}/hr^{rh\text{-}J}$). *Bottom panel*, Western analysis of same blot with β-tubulin antiserum to show equal loading of protein. Molecular mass (in kilodaltons) is indicated on the *left*. B, Hr coimmunoprecipitates with TR from

corepressor complex. We report that a HDAC inhibitor impairs Hr-mediated repression, suggesting Hr mediates repression through associated HDAC activity. Analysis of *hr* expression revealed that *hr* is widely expressed in neonatal rat brain and overlaps with multiple HDACs and other corepressors. TH-regulated expression of Hr in cell types and brain regions known to be TH sensitive, together with its function as a corepressor, implicates Hr as an important mediator of TH action in the developing brain.

RESULTS

Hr Associates with TR and HDACs in Brain

Our previous biochemical analysis indicates that Hr functions as a nuclear receptor corepressor (17). Hr can mediate transcriptional repression through direct interaction with unliganded TR. In addition, Hr associates with overexpressed HDACs and colocalizes with HDACs in cultured cells (17). To determine whether Hr might function as a corepressor in developing brain, we examined whether endogenous Hr interacts with TR and HDACs in brain. To facilitate the study of endogenous Hr, we generated an antibody to Hr (Fig. 1A). Using Western analysis on extracts prepared from rat cerebellum, the Hr antiserum detects a band of approximately 135 kDa, similar to the predicted size of rat Hr (130 kDa) (Fig. 1A). To confirm the specificity of the Hr antiserum, we used *hr* mutant mice ($hr^{rh\text{-}J}$ allele), which show more than 95% reduction of *hr* transcript and presumably lack Hr protein (Thompson, C. C., unpublished observations). Using Western analysis with Hr antiserum, the 135-kDa Hr band is detected in cerebellar extracts prepared from heterozygous but not homozygous *hr* mutant mice (Fig. 1A). Therefore, this antiserum detects endogenous Hr and was used to analyze Hr expression.

To determine whether endogenous Hr and TR interact in brain, we performed coimmunoprecipitation (CoIP) analysis on nuclear extracts prepared from postnatal d 21 (p21) rat cerebellum. Extracts were used for immunoprecipitation with TR-specific antiserum, followed by Western analysis with Hr-specific antiserum. Western analysis shows that when TR is immunoprecipitated, Hr is detected (Fig. 1B). Therefore, Hr coprecipitates with TR, indicating that these proteins interact *in vivo*. Previous experiments have indicated that Hr can associate with multiple HDACs in transfected cells (17). To examine whether Hr associates with HDACs under physiological conditions, we performed CoIP analysis on cerebellar nuclear extracts. Antisera recognizing various HDACs were used for immunoprecipitation followed by Western analysis with Hr-specific antiserum. We find Hr coimmunoprecipitates with HDAC1 and HDAC3, but not with HDAC2 (Fig. 1C). These results support previous experiments using overexpressed proteins and suggest that Hr associates with particular HDACs *in vivo*.

Previous experiments have shown that Hr can enhance repression by unliganded TR (17). The association of Hr with HDACs, together with data showing that transcriptional repression by other corepressors is dependent on HDAC activity, suggests that Hr-mediated repression occurs through associated HDAC activity (22–24). To test whether HDAC activity is required for repression by Hr, we assayed transcriptional activity in the presence of a potent HDAC inhibitor, trichostatin A (TSA) (Fig. 2A) (25, 26). Cultured pituitary cells endogenously expressing TR were transiently transfected with TH-responsive (MLVx2 tk-luc) or control (tk-luc) reporter constructs, and transcription was quantified by measuring luciferase activity (17, 27). When Hr is introduced by cotransfection of an expression vector, TH-responsive reporter gene activity is reduced 6-fold, indicating that Hr enhances transcriptional repression by unliganded TR. If HDAC activity is required for this repression by Hr, inhibiting HDAC activity with TSA should impair the ability of Hr to repress transcription through unliganded TR. Indeed, in the presence of TSA, repression by Hr is reduced by approximately 2-fold (Fig. 2A). In contrast, TSA has no effect on transcriptional activity of the TH-responsive reporter in the absence of Hr. Thus, inhibition of HDAC activity by TSA results in a significant reduction in repression by Hr, indicating that Hr-mediated repression is due, in part, to HDAC activity.

Previous work demonstrating the role of Hr as a corepressor that acts through TR suggests that Hr influences TH-responsive gene expression in the brain (17). To help determine whether Hr can mediate repression through TR on endogenous genes, we constructed and tested reporter genes using the TH-response elements (TREs) from two well characterized cerebellar genes, myelin basic protein (MBP)

brain. *Left*, Rat cerebellar nuclear extracts were immunoprecipitated with TR-specific antiserum or control mouse IgG (mIgG) as indicated. Hr was detected by Western analysis with Hr antiserum. *Right*, Western analysis of rat cerebellar nuclear extract with TR-specific antiserum. In, 7% of extract used for immunoprecipitation. C, Hr coimmunoprecipitates with HDACs from brain. *Left*, Rat cerebellar nuclear extracts were immunoprecipitated with HDAC-specific antisera or control rabbit IgG (rIgG) as indicated. Hr was detected by Western analysis with Hr antiserum. *Right*, Western analysis of rat cerebellar nuclear extracts with HDAC antisera (α-HD1, HDAC1-specific antiserum; α-HD2, HDAC2-specific antiserum; α-HD3, HDAC3-specific antiserum). "In" indicates 7% of extract used for immunoprecipitation. Molecular mass (in kilodaltons) is indicated on the *left*. D, Model of Hr corepressor complex. TR and its typical heterodimerization partner retinoid X receptor (RXR) bind to a TRE upstream of a TH-responsive gene. In the absence of TH, Hr associates with TR, HDACs, and likely other proteins (*smaller, unlabeled shapes*) as a corepressor complex to repress transcription.

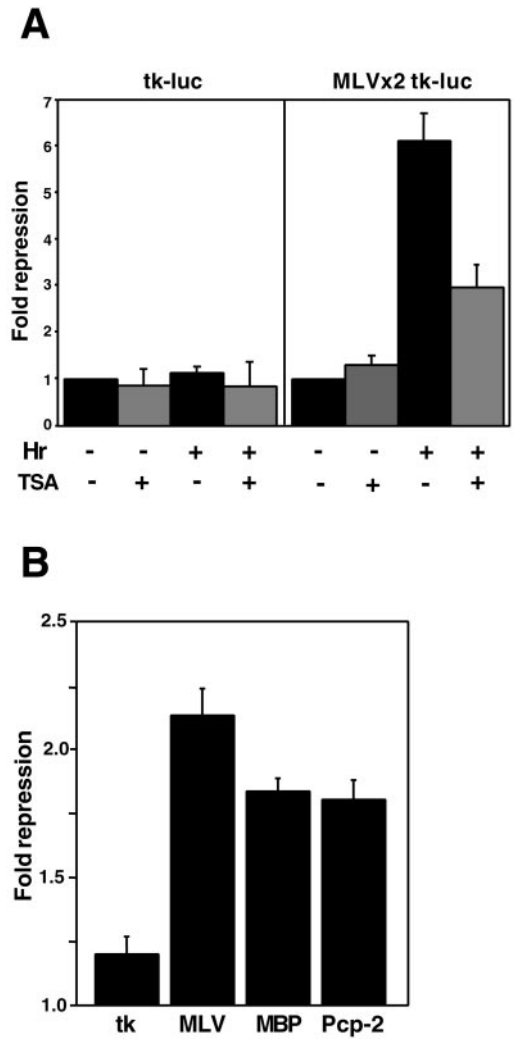

Fig. 2. Hr Mediates Transcriptional Repression by Unliganded TR via HDAC Activity

A, TH-responsive (MLVx2 TRE tk-luc, has two copies of the TRE from the MLV LTR) and control (tk-luc) reporter genes were cotransfected into cultured pituitary (GH1) cells with an expression vector for Hr or vector alone. Luciferase activity was measured in the absence of TH and the absence or presence of 100 nM TSA, a HDAC inhibitor. Fold repression is luciferase activity of the reporter gene alone divided by luciferase activity of the reporter gene in the presence of Hr, TSA, or both, as indicated. Hr-mediated repression is reduced approximately 2-fold by TSA. Shown are the mean values ± SD of five independent experiments done in duplicate. B, Hr can repress transcription via multiple TREs. Cultured pituitary cells (GH1, GC) were transfected with reporter genes under the control of various TREs. tk-luc, Negative control; MLV, MLV tk-luc (one copy of the MLV TRE); MBP, MBP tk-luc (one copy of the TRE from the myelin basic protein gene); Pcp-2, Pcp-2 tk-luc (one copy of the 5′-upstream TRE (A1) from the Purkinje cell protein-2 gene). Fold repression is luciferase activity of the reporter gene alone divided by luciferase activity of the reporter gene in the presence of Hr. Shown are the mean values ± SE of eight independent experiments done in duplicate.

and Purkinje cell protein 2 (Pcp-2) (28, 29). A single copy of the MBP or Pcp-2 TRE was inserted upstream of a minimal thymidine kinase (tk) promoter, and these reporter genes were introduced into cells expressing endogenous TR. Repression by unliganded TR was measured in the absence and presence of cotransfected expression vector for Hr (Fig. 2B). A reporter gene with a single TRE from a viral promoter [Moloney murine leukemia virus (MLV) long terminal repeat] previously shown to support repression by TR:Hr (17) showed about a 2-fold decrease in transcriptional activity in the presence of Hr. Similarly, expression of Hr resulted in decreased transcription from the MBP and Pcp-2 reporter genes. No significant repression was observed with the reporter gene lacking a TRE, suggesting that Hr-mediated repression is TR dependent. In addition, repression was specific for TR-bound Hr, as reporter gene expression was not affected by expression of a mutant Hr (amino acids 31–568) that lacks the TR binding domains (data not shown). Thus, Hr can mediate transcriptional repression by unliganded TR on TREs from endogenous TH-responsive genes, suggesting that Hr may function in a similar manner *in vivo*.

Hr Is Broadly Expressed in Neonatal Rat Brain

To identify potential sites of *hr* action *in vivo*, we determined the regional localization of *hr* expression at the cellular level. We have shown previously that *hr* is expressed in whole brain by Northern analysis and in cerebellum by *in situ* hybridization (11). To identify other brain regions that express *hr*, we performed *in situ* hybridization using *hr*-specific antisense cRNA probes on parasagittal and coronal sections from p15 rat brain (Fig. 3). Hybridization with a control sense probe did not produce a signal (Fig. 3A and data not shown). Expression of *hr* was detected in several brain regions with particularly high expression in the cortex, hippocampus, thalamus, and cerebellum. *hr* is also expressed in the cochlear nucleus and inferior colliculus of the auditory system. In addition, *hr* is expressed in several fiber tracts including the corpus callosum, optic tract, internal capsule, and cerebral peduncle, indicating expression may also be in glial cells (Fig. 3, A and B).

Because Hr functions in a manner analogous to the well characterized corepressors SMRT and N-CoR, we examined whether *hr* expression overlaps with that of SMRT and N-CoR in neonatal rat brain (Fig. 4). *In situ* hybridization of coronal sections from p15 rat brain revealed that both SMRT and N-CoR are widely expressed in the brain. Both are detected in cortex, hippocampus, amygdala, thalamus, and piriform cortex. The pattern of expression includes regions that do not express *hr* such as amygdala. Expression of all three corepressors overlaps in multiple regions of the brain including hippocampus (CA3 and dentate gyrus) and cortex.

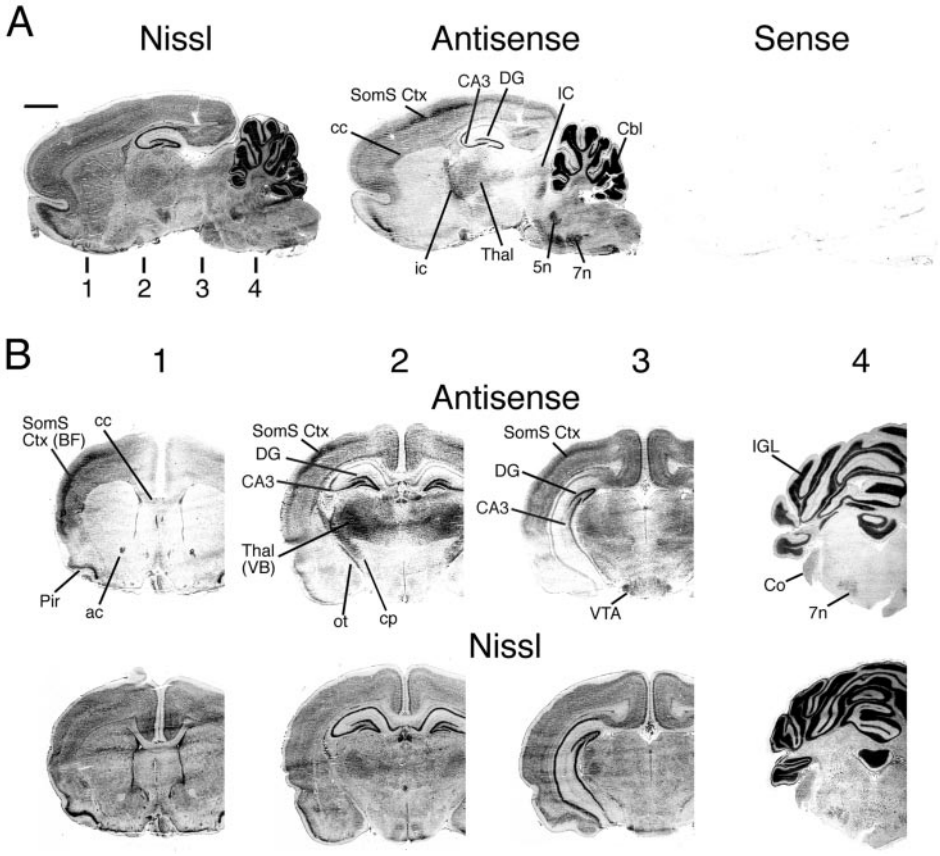

Fig. 3. *hr* Is Broadly Expressed in Developing Rat Brain

A, *In situ* hybridization with *hr*-specific probes on parasagittal p15 rat brain sections. Nissl, Cresyl violet stain to identify cell bodies; antisense, *in situ* hybridization with antisense *hr*-specific probe; sense, *in situ* hybridization with sense control probe. *Numbers in left panel* indicate rostral-caudal level of coronal sections in panel B. *Scale bar,* 2 mm. B, *In situ* hybridization on p15 coronal brain sections with *hr*-specific probe. *Top panels*, Serial sections used for *in situ* hybridization with antisense *hr*-specific probe; sense control probe did not produce a detectable signal (data not shown). *Bottom panels*, Cresyl violet-stained serial sections 60–120 μm from corresponding *in situ* hybridized section to identify cell bodies. *Numbers above top panels* indicate rostral-to-caudal plane of section shown in panel A. BF, Barrel field; CA3, CA3 region of hippocampus; Cbl, cerebellum; Co, cochlear nucleus; DG, dentate gyrus of hippocampus; IC, inferior colliculus; IGL, internal granule cell layer of cerebellum; Pir, piriform cortex; SomS Ctx, somatosensory cortex; Thal, thalamus; VB, ventrobasal nuclear complex; VTA, ventral tegmental area; 5n, trigeminal nucleus; 7n, facial nucleus; ac, anterior commissure; cc, corpus callosum; cp, cerebral peduncle; ic, internal capsule; ot, optic tract.

hr and *hdac* Expression Overlap *in Vivo*

Results of CoIP analysis (Fig. 1) show that Hr, TR, and HDACs interact in cerebellum. To identify other brain regions in which these proteins potentially associate *in vivo*, we addressed whether expression of these proteins overlaps within the brain. The localization and expression of both TR genes (TRα and TRβ) and their isoforms within the brain are well documented (30, 31). Both TRs are highly expressed in the neocortex, hippocampus, and cerebellum during neonatal development (30, 32). The expression of *hr* closely correlates with brain regions that express high levels of TRs, suggesting that the interaction we have detected *in vitro* occurs in these regions *in vivo*. Whereas the localization of TRs has been determined, the regional expression of the HDACs within the brain has not yet been established. We determined whether *hr* and *hdac* expression overlap in brain by analyzing the expression of *hdac3* and *hdac5* by *in situ* hybridization on sections prepared from neonatal rat brain. These HDACs were chosen for analysis because they interact with Hr by CoIP analysis and have been implicated as key components of corepressor complexes (17, 22, 33–36). In agreement with CoIP experiments that indicate an association between Hr and HDACs in cerebellum, *in situ* hybridization analysis on coronal cerebellar sections from p15 rat show that *hr*, *hdac3*, and *hdac5* are all highly expressed in the internal granule cell layer of the cerebellum (Fig. 5A). To identify other regions of overlapping expression between *hr* and *hdac* in the brain, we performed *in situ* hybridization on coronal forebrain sections prepared from p15 rat. Within forebrain, *hr*, *hdac3*, and *hdac5* show coinci-

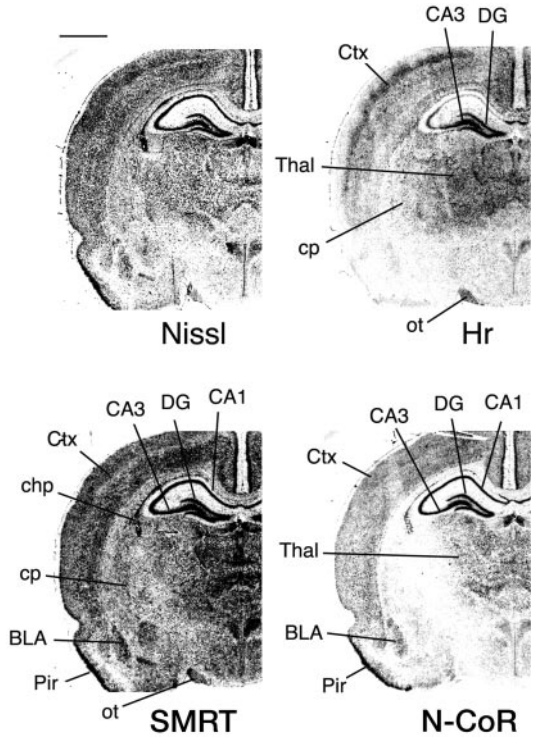

Fig. 4. *hr* Expression Overlaps with that of Other Corepressors

In situ hybridization on serial, coronal sections from p15 rat forebrain with probes specific for *hr*, SMRT, and N-CoR. Nissl, Cresyl violet stain to identify cell bodies; *hr*, SMRT and N-CoR expression overlaps in several regions. Ctx, Cortex; CA1 and CA3, CA1 and CA3 regions of hippocampus; DG, dentate gyrus of hippocampus; Thal, thalamus; cp, caudate putamen; ot, optic tract; chp, choroid plexus; BLA, basal lateral nucleus of the amygdala; Pir, piriform cortex. *Scale bar,* 2 mm.

dent expression in the cortex, hippocampus, thalamus, and hypothalamus, demonstrating the potential for *in vivo* interaction (Fig. 5B). *hdac1* expression was also analyzed and appears uniformly expressed in the brain (data not shown).

To further identify potential sites of Hr action *in vivo*, we next wanted to examine the spatial and temporal pattern of Hr expression. By determining where and when Hr is expressed, we will begin to understand the tissues and developmental periods in which Hr exerts its biochemical effects. To determine which tissues express Hr, protein extracts were prepared from p15 rat and examined for Hr expression by Western analysis. Consistent with previous Northern analysis (11), Hr is highly expressed in the brain and skin with lower levels detected in lung and pituitary (Fig. 6, A and B). To examine which brain regions express Hr, we performed Western analysis on extracts prepared from various regions of the p15 rat central nervous system. In agreement with *in situ* hybridization results, we find that Hr is highly expressed in the cerebellum, somatosensory cortex, inferior colliculus, olfactory bulb, thalamus, and optic tract. In addition, we detect Hr expression in the spinal cord (Fig. 6B).

Hr Expression Is Influenced by TH in Brain

Previous work has shown that expression of the *hr* transcript is rapidly and directly induced by TH in cerebellum (2, 11). To examine the dependence of *hr* expression by TRs, we analyzed *hr* expression in TR null mice (Fig. 7). Northern analysis was performed using total RNA isolated from the brains of mice that lack expression of one or more TR isoforms (37–40). Mice lacking TRβ1 showed no change in *hr* expression in the brain. In contrast, *hr* expression was significantly lower in both the TRα- and TRα1-deficient animals. Expression of *hr* in mice null for both TRα and TRβ1 was lower as well, although some expression remained. Thus, TH-regulated expression of *hr* likely occurs primarily through TRα.

To determine whether Hr protein levels are similarly affected by TH levels in brain, we examined the kinetics of Hr expression in the cerebellum in response to TH. Hypothyroid animals were treated with TH for various periods of time, cerebellar extracts were prepared, and Hr expression was determined by Western analysis. Hr is detected within 3 h of TH administration and reaches normal, euthyroid levels within 12 h (Fig. 8A). Thus, Hr expression increases rapidly upon stimulation by TH, supporting the notion that *hr* expression is directly regulated by TH in the brain.

Because TH exerts its effects during a critical period of development in the cerebellum that extends from the first to third postnatal weeks, we next analyzed Hr expression during this period. Cerebellar extracts were prepared from rats ranging in age from birth (p0) to adult and analyzed for Hr expression by Western analysis. Hr is detected by p10 and reaches a plateau by p13. Expression remains relatively high during the second to fourth postnatal weeks and eventually decreases in the adult (Fig. 8B). This analysis shows that Hr is developmentally regulated and its expression correlates with the critical period of TH action in cerebellum. Because TH levels rise at birth and Hr expression is regulated by TH, Hr expression is likely induced by TH during this time in development. If this is the case, then reducing the level of TH should likewise reduce the level of Hr during this period. Accordingly, we analyzed Hr expression by Western analysis on extracts prepared from the cerebellum and cortex of euthyroid and hypothyroid animals ranging in age from p5 to adult (Fig. 8, C and D). In the euthyroid rat cerebellum, expression of Hr is detected by p10, peaks around p20, and is maintained at a lower level in adult. In contrast, in the hypothyroid rat cerebellum, Hr is not detected until p25 and remains at a low level into adulthood (Fig. 8C). Similar to the cerebellum, Hr is developmentally regulated in the somatosensory cortex. In the euthyroid animal, Hr expression is detected at p5, peaks around p10, remains high during the first 3 postnatal weeks, and decreases in the adult. In the

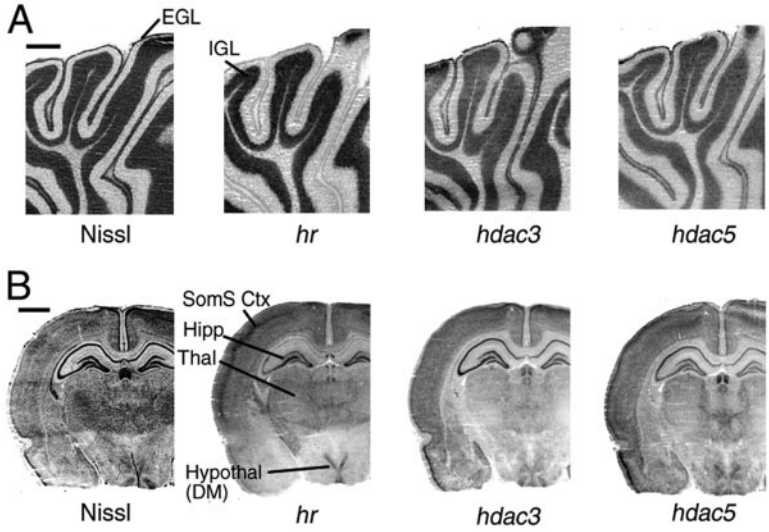

Fig. 5. *hr* and *hdac* Expression Overlap in Neonatal Rat Brain

A, *In situ* hybridization was performed on serial, coronal sections from p15 rat cerebellum with the indicated probes. Note overlapping expression of *hr* and *hdacs* in internal granule cell layer (IGL) of cerebellum. EGL, External granule cell layer; Nissl, cresyl violet stain to identify cell bodies. *Scale bar,* 500 μm. B, *hr* and *hdac* expression overlap in neonatal rat forebrain. *In situ* hybridization performed on serial, coronal sections from p15 rat forebrain with the indicated probes. *hr* and *hdac* expression overlap in several regions including somatosensory cortex (SomS Ctx), hippocampus (Hipp), thalamus (Thal), and dorsomedial nucleus of hypothalamus [Hypothal (DM)]. Nissl, Cresyl violet stain to identify cell bodies. *Scale bar,* 2 mm.

hypothyroid somatosensory cortex, Hr expression is lower overall at the developmental ages examined (Fig. 8D).

To address potential regional differences or local cellular contributions of TH-regulated *hr* expression, we performed *in situ* hybridization on brain sections prepared from euthyroid and hypothyroid rats (Fig. 9). Since we have previously shown that *hr* expression is greatly reduced in hypothyroid cerebellum, we analyzed the effect of TH on *hr* expression in rat forebrain (11). In coronal sections of euthyroid rat forebrain, we find that *hr* is highly expressed in the cortex, hippocampus, and thalamus. In the hypothyroid rat brain, *hr* expression was diminished in almost all regions examined including somatosensory cortex, hippocampus, and thalamus (Fig. 9A). In the somatosensory cortex, *hr* is broadly expressed with highest expression in layer 4 (Fig. 9B). Strikingly, *hr* expression is greatly reduced in all cortical layers within the hypothyroid brain except for a population of cells in layer 6. Within the hippocampus, *hr* is highly expressed in the dentate gyrus and CA3 regions, while in the hypothyroid hippocampus, expression of *hr* is reduced in these regions (Fig. 9B). In addition, *hr* expression appears unchanged in the optic tract, suggesting *hr* expression may be TH-independent in glial cells (Fig. 9C).

DISCUSSION

Hr Is a Nuclear Receptor Corepressor in Brain

Our previous *in vitro* studies demonstrated that Hr has the biochemical properties of a nuclear receptor corepressor: Hr enhances repression by unliganded TR, contains multiple autonomous repression domains, and interacts with HDACs (17). Using Hr-specific antiserum, we now show that endogenous Hr and TR interact under physiological conditions. The association detected between Hr and TR by CoIP using cerebellar nuclear extracts suggests that Hr and TR also associate in other regions of the brain where they are coincidentally expressed, such as cortex and hippocampus.

Other nuclear receptor corepressors that interact with TR and retinoic acid receptors have been shown to form multiprotein complexes that include several HDACs (34, 41, 42). HDACs are thought to be critical components of corepressor complexes because HDAC enzymatic activity is required for repression (22, 23, 42–45). Consistent with results using overexpressed proteins, we show here that endogenous Hr associates with HDACs 1 and 3 under physiological conditions. Analysis of *hr* and *hdac* expression in the brain by *in situ* hybridization shows that expression of *hr* and *hdac* largely overlaps, indicating the potential for extensive *in vivo* interaction. Hr association with HDACs appears to be functionally significant, as inhibition of HDAC activity by a specific HDAC inhibitor (TSA) reduces the ability of Hr to cause repression by unliganded TR. Histone deacetylation is thought to cause repression by altering chromatin structure; additional experiments using stably integrated reporter genes will be necessary to assay whether the Hr-HDAC complex acts through this mechanism. Inhibition of HDAC activity by TSA does not abolish Hr-mediated repression, suggesting that HDAC activity is

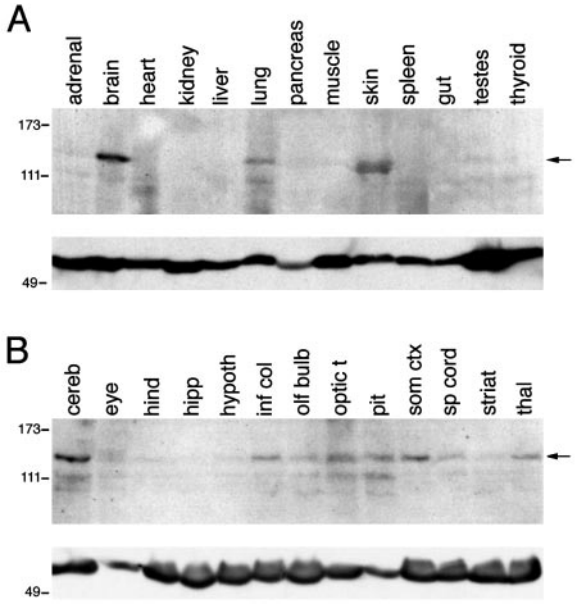

Fig. 6. Hr Expression Is Tissue Restricted

A, *Top panel*, Western analysis with Hr antiserum on protein extracts from the indicated tissues of p15 rat. *Bottom panel*, Western analysis of same blot with β-tubulin antiserum to show equivalent loading of protein. B, Hr is broadly expressed in neonatal rat brain. *Top panel*, Western analysis on protein extracts from various tissues from the p15 rat nervous system. *Bottom panel*, Western analysis of same blot with β-tubulin antiserum to show equal loading of protein. *Arrows* indicate position of Hr protein; molecular mass (in kilodaltons) is indicated on the *left*. cereb, Cerebellum; eye, whole eye; hind, hindbrain; hipp, hippocampus; hypoth, hypothalamus; inf col, inferior colliculus; olf bulb, olfactory bulb; optic t, optic tract; pit, pituitary; som ctx, somatosensory cortex; sp cord, spinal cord; striat, striatum; thal, dorsal thalamus.

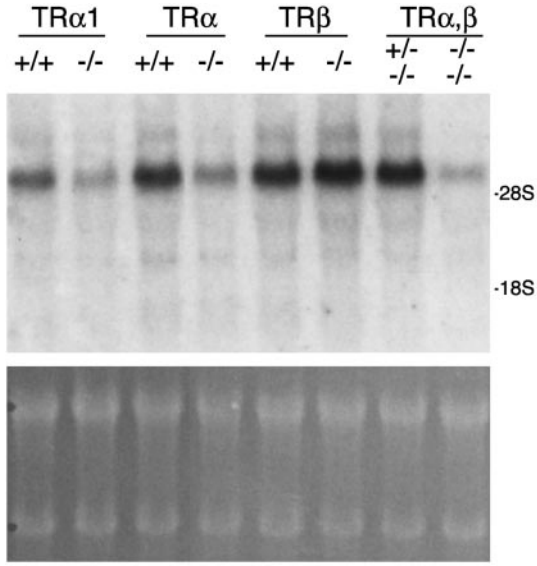

Fig. 7. Expression of *hr* Is Regulated by TRα

Northern analysis of total RNA isolated from the brains of mice lacking expression of the indicated TR isoforms. *Upper panel*, Autoradiograph of Northern blot with radiolabeled *hr*-specific probe; *lower panel*, ethidium bromide-stained gel to show equivalent loading of RNA. $+/+$, Wild type; $-/-$, homozygous gene deletion. TRα1, Deletion of TRα1 isoform only (39); TRα, deletion of entire TRα locus (38); TRβ1, deletion of TRβ1 isoform (37); TRα, β1, deletion of entire TRα locus and TRβ1 isoform (40).

necessary, but not sufficient, for maximal repression and that HDAC-independent mechanisms may be used as well. HDAC-independent mechanisms for repression have been implicated for other nuclear receptor corepressors, as N-CoR and SMRT interact with the basal transcription factor TFIIB and may inhibit transcriptional activation by interfering with the preinitiation complex (46, 47).

What is the functional significance of multiple HDACs (17) associating with a single nuclear receptor corepressor? Studies examining HDAC function have shown that the activity of type II HDACs (HDAC4 and HDAC7) is linked to their interaction with a type I HDAC (HDAC3), suggesting that a combination of HDACs might be necessary for producing an enzymatically active complex (48, 49). Other experiments have shown that HDACs 4 and 5 interact with HDAC3 (50). Our investigation of *hdac* expression shows overlapping regional localization within brain between *hdac3* and *hdac5*, suggesting a potential role for cooperation between these HDACs. Synergy between multiple HDACs might be necessary for maximal HDAC activity and consequently effective repression of gene transcription.

Hr as a Mediator of TH Action

Hr resembles other corepressors (SMRT and N-CoR) in its association with TR and HDACs. Analysis of corepressor expression in neonatal rat brain shows that *hr*, SMRT, and N-CoR are coexpressed in multiple brain regions including cortex, hippocampus, thalamus, and cerebellum (Fig. 4 and Refs. 51 and 52). The expression of multiple corepressors in the same cell populations raises the question of whether corepressors act cooperatively by recruiting factors to a shared complex or compete for binding to nuclear receptors. The unique spatial and temporal expression of Hr suggests it might confer distinct properties to the corepressor complex not provided by more ubiquitous corepressors. In addition, Hr interacts with TR and not retinoic acid receptor, and recruits a particular complement of HDAC proteins (12, 17).

The regulation of *hr* expression by TH is unique among nuclear receptor corepressors (11, 51, 53). We show here that expression of *hr* is probably dependent on the TRα isoform, as *hr* levels decrease in mice lacking TRα but are unchanged in mice lacking TRβ. However, regulation of *hr* expression is likely complex, as deletion of both TRs does not abolish expression, indicating that non-TR-dependent mechanisms regulate *hr* expression as well.

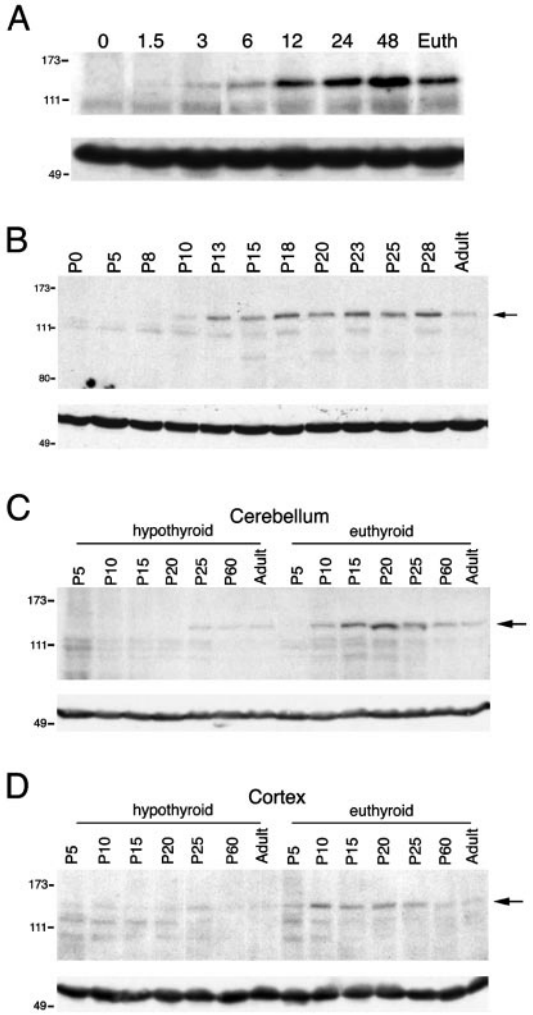

Fig. 8. Hr Expression Is Regulated by TH During Development

A, Hr is rapidly expressed after administration of TH. *Top panel*, Western analysis of extracts prepared from cerebellum of p15 hypothyroid rats treated with TH for the number of hours indicated above blot. 0, Hypothyroid control; Euth, normal, euthyroid control. *Bottom panel*, Western analysis of same blot with β-tubulin antiserum to show equal loading of protein. B, Hr expression is developmentally regulated in cerebellum. *Top panel,* Cerebellar extracts prepared from rats ranging in age from birth (P0) to adulthood were examined for Hr expression by Western analysis. *Bottom panel,* Western analysis of same blot with β-tubulin antiserum to show equal loading of protein. C, Effect of TH on Hr expression during cerebellar development. *Top panel*, Western analysis with Hr antiserum on extracts prepared from cerebellum of hypothyroid or euthyroid (untreated) rats at the postnatal ages indicated. *Bottom panel*, Western analysis of same blot with β-tubulin antiserum to show equal loading of protein. D, Effect of TH on Hr expression during postnatal cortical development. *Top panel*, Western analysis with Hr antiserum on extracts prepared from somatosensory cortex collected from hypothyroid or euthyroid (untreated) rats at the postnatal ages indicated. *Bottom panel*, Western analysis of same blot with β-tubulin antiserum to show equal loading of protein. *Arrow* in panel B–D indicates position of Hr protein. Molecular mass (in kilodaltons) is indicated on the *left*.

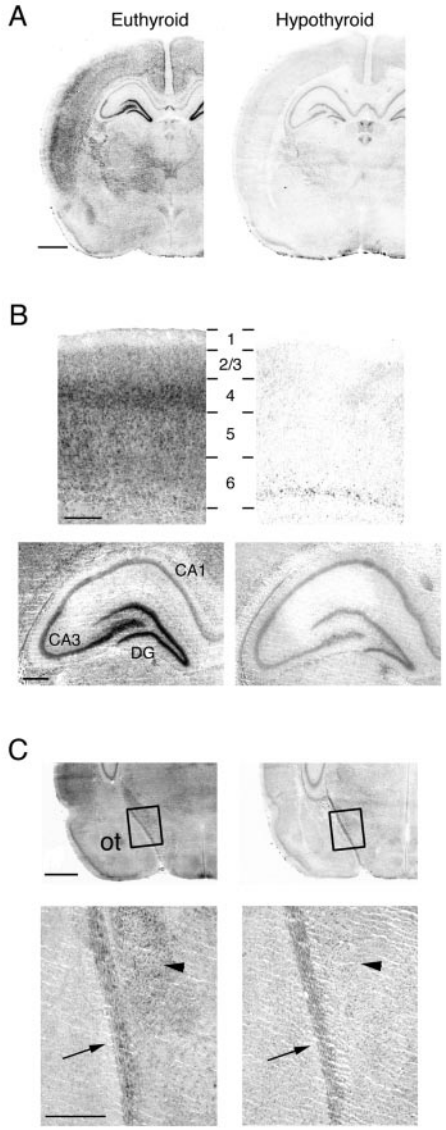

Fig. 9. hr Is Differentially Regulated by TH in Rat Brain

A, *hr* Expression is reduced in hypothyroid neonatal rat brain. Low-magnification image of *in situ* hybridization performed on coronal sections from euthyroid (*left panel*) or hypothyroid (*right panel*) p15 rat brain with a *hr*-specific probe. B, *hr* is regulated by TH in cortex and hippocampus. *Upper panels*, Higher-magnification images of sections in panel A showing *in situ* hybridization on euthyroid (*left*) or hypothyroid (*right*) somatosensory cortex. *Numbers (1–6)* indicate cortical layers. *Bottom panels*, Higher magnification images of sections in panel A showing *in situ* hybridization on euthyroid (*left*) or hypothyroid (*right*) hippocampus. CA1, CA1 region; CA3, CA3 region; DG, dentate gyrus. C, *hr* expression in optic tract (ot) is unaffected by TH. *Top panels*, Low-magnification images of *in situ* hybridization with *hr*-specific probes on a more caudal coronal section than shown in panel A. *Boxed region* is shown at higher magnification *below*. *Lower panels*, Higher magnification of *in situ* hybridization on euthyroid (*left*) and hypothyroid (*right*) sections showing similar expression levels of *hr* in the optic tract. *Arrow* points to optic tract; *arrowhead* points to thalamic nuclei that show TH-dependent *hr* expression. *Scale bar* in A and C (*top panel*), 2 mm; B and C (*bottom panel*), 500 μm.

After TH increases *hr* expression, followed by a concomitant increase in Hr protein levels, Hr presumably binds to TR and modulates its activity, suggesting a potential autoregulatory pathway. *In vitro* studies show that Hr binds to TR preferentially in the absence of TH, raising the question of whether Hr interacts with TR *in vivo* (17). In this study, we detect Hr-TR interaction in extracts from euthyroid brain, indicating Hr and TR can associate when the animal has normal TH levels. The apparent discrepancy may be due to TH concentration, as *in vitro* studies typically use the active form of TH, T_3, at concentrations 100- to 1000-fold higher than physiological levels ($\sim 10^{-9}$ M) (17–19, 54). Local T_3 concentration may be important *in vivo*, as T_3 levels can vary between brain regions, and the activity of both activating (types I and II) and deactivating (type III) deiodinases can modulate concentrations of T_3 at the cellular level (55–57).

Hr Action *in Vivo*

TH influences a number of developmental processes in the brain, presumably by initiating a program of gene expression. As a TH-responsive gene, *hr* is a component of this genetic program. As a corepressor that can influence TR function, Hr likely has a role in regulating the expression of downstream TH-responsive genes. We find that Hr can mediate repression by unliganded TR through the TREs of endogenous TH-responsive genes (MBP, Pcp-2), suggesting an *in vivo* role as a transcriptional repressor. Experiments directly demonstrating Hr-dependent regulation of TH-responsive gene expression *in vivo* awaits both the identification of TH-responsive genes that are expressed in the same cell types as Hr, as well as generation of animals with an *hr* null allele, as existing alleles maintain some *hr* expression (Thompson, C. C., unpublished observations).

Other factors, including other nuclear receptors, have been shown to repress TH-responsive gene expression. For example, the orphan receptor chicken ovalbumin upstream promoter transcription factor (COUP-TF) has been shown to repress TH-regulated expression of the Pcp-2 gene in Purkinje cells (58). The mechanism by which COUP-TF represses Pcp-2 expression is distinct from repression by Hr, as COUP-TF is thought to compete with TR for DNA binding and thereby interfere with TH-activated transcription. However, COUP-TF has an independent repression domain that interacts with other corepressors (N-CoR, SMRT) (59); it will be of interest to determine whether Hr can interact with COUP-TF as well.

The phenotypic characteristics of TH deficiency in the brain are not readily detectable at a gross morphological level (60, 61). Similarly, profound morphological alterations that result from loss of *hr* expression in the nervous system have not been reported. Although existing *hr* mutant alleles are not null, several phenotypes described for *hr* mutant mice resemble phenotypes associated with TH deficiency. For example, alterations in the size of Purkinje cells have been reported for *hr* mutant mice and TH-deficient animals (8, 15, 62). In addition, *hr* ($hr^{rh\text{-}J}$ allele) mutant mice exhibit cochlear defects similar to congenital hypothyroid ($Tshr^{hyt}$) mutant mice and human TH deficiency disorders (16, 63, 64). Cochlear defects also correlate with TR expression in the inner ear and hearing deficiencies in TRβ null mice (65, 66). Expression of *hr* within the cerebellum and auditory system reported here indicates such changes may be the result of loss of Hr function and consequent disruption of TH-responsive gene regulation.

In general, morphological alterations that result from loss of *hr* expression in the nervous system have remained largely unexplored. Our analysis of *hr* expression in the brain provides a map to brain regions in which morphological alterations in *hr* mutant mice might be detected. For example, we detect expression of *hr* within the somatosensory cortex, hippocampus, and several fiber tracts, suggesting that these structures might be influenced by loss of Hr function. Interestingly, these regions are sensitive to TH deficiency, as in hypothyroid brain both the somatosensory cortex and hippocampus show a reduction in dendritic growth and synaptogenesis (reviewed in Refs. 1 and 67).

Detection of *hr* mRNA in multiple fiber tracts indicates that *hr* is expressed in glial cells as well as neurons. TH effects on glial cells include regulation of oligodendrocyte differentiation and maturation of astrocytes (6, 68). Intriguingly, expression of glial fibrillary acidic protein, a marker of astrocyte maturation, is reduced in the brains of both *hr* mutant mice ($hr^{rh\text{-}J}$ allele) and hypothyroid animals, suggesting a link between *hr* function and TH action and implying a possible role for *hr* in astrocyte differentiation (69–71). Unlike most brain regions, *hr* expression was unchanged in the optic tract of hypothyroid animals, suggesting that regulation of *hr* expression may differ between neurons and glia.

Our analysis of *hr* function and expression supports the notion that *hr* has a role in mediating the effects of TH on brain development. Although it is likely that TH regulates the expression of many genes in developing mammalian brain, the function of the Hr protein as a corepressor for TR emphasizes that *hr* is an integral component of TH action.

MATERIALS AND METHODS

Generation of Hr-Specific Antiserum

Amino acids 450–730 of rat Hr were expressed as a glutathione-*S*-transferase fusion protein in bacterial strain DH10. Protein was separated by SDS-PAGE and acrylamide containing the fusion protein was used to immunize rabbits (Covance Laboratories, Inc., Denver, PA). Antiserum was affinity purified using a bacterially expressed fusion protein of Hr amino acids 450–730 and trpE.

Animal Care

Hairless mice (RHJ/LeJ $hr^{rh\text{-}J}$) were obtained from The Jackson Laboratory (Bar Harbor, ME), and Sprague Dawley rats were obtained from Charles River Laboratories, Inc. (Wilmington, MA). To induce TH deficiency in neonatal rat pups, pregnant Sprague Dawley rats were treated from d 13 of gestation by administration of 0.025% methimazole (Sigma, St. Louis, MO) in their drinking water. We have used this method previously to reduce TH levels and TH-responsive gene expression in neonatal rats (11, 72). Animal care and treatment were in accordance with NIH guidelines and were approved by the Johns Hopkins University School of Medicine Institutional Animal Care and Use Committee.

Western Analysis of Hr Protein Expression

Protein sample preparation: To analyze Hr expression in *hr* mutant mice, cerebellar tissue was collected and pooled from at least three male p28 heterozygous ($hr^{rh\text{-}J}/+$) or homozygous ($hr^{rh\text{-}J}/hr^{rh\text{-}J}$) mutant mice. For kinetic study of Hr expression, hypothyroid rats were injected sc with 0.25 μg/g body weight L-T_3 (Sigma) at 48, 24, 12, 6, 3, or 1.5 h before animals were killed at p15. One group of hypothyroid animals (0 in Fig. 6A) received injections of saline instead of L-T_3 for 48 h. Animals treated for 48 h were injected once every 24 h. Cerebella were dissected and pooled from at least three animals per time point. For the tissue distribution, tissues were dissected and pooled from at least four p15 animals, two males and two females. Hippocampus, striatum, hypothalamus, dorsal thalamus, and cerebellum were dissected as described (73, 74). Other tissues and organs were identified by morphological criteria. For the developmental study, the cerebellum or somatosensory cortex of two male and two female rats ranging in ages from birth (p0) to adult (p98–p105) were dissected and pooled (per brain region and time point). An identical procedure was used to prepare cerebellar and cortical extracts from hypothyroid rats.

For Western blot analysis, dissected tissue samples were weighed, homogenized using 50 mg of tissue per milliliter of 1× SDS-PAGE sample buffer, and boiled for 3 min. Protein concentration was estimated by SDS-PAGE in comparison with a protein extract of known concentration. Approximately 60 μg of each protein preparation were loaded per lane. Proteins were separated by SDS-PAGE, transferred to Hybond-P polyvinylidine difluoride membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK), and analyzed by Western blot analysis using affinity-purified Hr-antiserum (1:100 dilution). Western analysis was performed at least three times with similar results. A representative Western blot is shown in Figs. 1, 6, and 8. All blots were reprobed with β-tubulin antiserum (Sigma) to control for equivalent loading of protein.

In Situ Hybridization

For the *hr*-specific probe, a fragment corresponding to nucleotides 387-2354 of the rat *hr* cDNA was subcloned into pBluescript KS+ (Stratagene, La Jolla, CA), and the resulting plasmid was linearized with *Not*I or *Sal*I to generate templates for sense or antisense probes, respectively. For the N-CoR-specific probe, a cDNA encoding rat N-CoR was obtained by PCR amplification of reverse-transcribed total RNA from rat heart and liver using specific primers (5′-CTAAGGACCCGAGGGAAGAC-3′, 5′-CTGAATGAGGTGATGGGTCC-3′) (Beaudoin, G., and C. C. Thompson, unpublished data). A *Pst*I–*Xba*I fragment of the rat N-CoR cDNA consisting of nucleotides 70–1067 (AF124821) was subcloned into pBluescript KS+. For the SMRT-specific probe, a cDNA encoding mouse SMRT (kindly provided by Dr. R. Evans, The Salk Institute, La Jolla, CA) was digested with *Bam*HI, and a fragment corresponding to nucleotides 5456–7078 (AF113001) was subcloned into pBluescript KS+. Probes for HDAC3 and HDAC5 were generated by PCR amplification of reverse-transcribed rat heart total RNA using specific primers (HDAC3, 5′-TTGAAGATGCTGAACCATGC-3′, 5′-AATGGACATAAAGCCGGTTG-3′; HDAC5, 5′-TTGTCCAAGTCAAAGGAGCC-3′, 5′-CTATGAGCGTGCTCTGTTGC-3′). Amplified fragments were subcloned into pCR2.1 (Invitrogen, San Diego, CA) in both orientations.

Sense and antisense digoxigenin-labeled cRNA probes were generated by *in vitro* transcription according to the specifications of the manufacturer (Roche, Welwyn Garden City, UK). All probes were hydrolyzed to an average size of 150 bp and used for *in situ* hybridization as described previously (72, 75). To visualize cell bodies, serial sections 60–120 μm from sections used for *in situ* hybridization were stained with 0.5% cresyl violet (Cellpoint Scientific, Rockville, MD) (Nissl stain in Figs. 3–5). Digital images of sections were captured using an Axiocam attached to an Axiophot or Stemi SV6 microscope (Carl Zeiss, Thornwood, NY). Digital images were processed using Adobe Photoshop (version 5.5) software (Adobe Systems Inc., San Jose, CA) by equally adjusting the brightness and contrast levels of sense and antisense images until background signal of sense images was undetectable. Remaining detectable signals in antisense images represent regions with transcript expression greater than background.

Northern Analysis

Total RNA was prepared from frozen brain tissue from the following TR null mice (ages indicated): TRα1 (p14), deletion of TRα1 isoform only (39); TRα (p21), deletion of entire TRα locus (38); TRβ1 (p16), deletion of TRβ1 isoform (37); TRα,β1 (p18), deletion of entire TRα locus and TRβ1 isoform (40). Total RNA (15 μg per lane) was separated on formaldehyde-agarose gels and transferred to nitrocellulose. Ethidium bromide staining was used to assure that equivalent amounts of RNA were loaded per lane, and filter was checked after blotting to confirm equivalent transfer of RNA. The filter was hybridized to a radiolabeled cDNA fragment corresponding to the full-length rat *hr*. After washing, filter was exposed to x-ray film.

Transcriptional Repression Assay

GH1 cells were obtained from the ATCC (Manassas, VA) and maintained in MEM supplemented with 10% fetal bovine serum, 0.1 mM nonessential amino acids (Life Technologies, Inc., Gaithersburg, MD), and 1 mM sodium pyruvate (BioWhittaker, Inc., Walkersville, MD). GC cells were maintained in DMEM supplemented with 10% horse serum and 5% fetal bovine serum. TH-depleted serum was produced by treatment with AG-1-X8 resin (Bio-Rad Laboratories, Inc., Hercules, CA) and charcoal (Sigma) as described previously (76). Cells were plated in hormone-depleted media at 400,000 cells per well of a 12-well plate 1 d before transfection. Cells were transfected overnight with 75 ng/well of CMX-β-galactosidase, 150 ng/well of reporter (tk-luc derivatives), and 90 ng/well of expression plasmid (pRK-myc derivatives) using Lipofectamine2000 (Invitrogen). After transfection, media was replaced with TH-depleted media and supplemented with 100 nM TSA (Sigma) as indicated (Fig. 2A). Cells were harvested 1 d later with passive lysis buffer (Promega Corp., Madison, WI) and assayed for β-galactosidase and luciferase activity. For each sample, luciferase activity was normalized to β-galactosidase activity. Fold repression was calculated by dividing luciferase activity of reporter alone by luciferase activity in the presence of cotransfected Hr expression vector. For the TSA experiment, fold repression was calculated by dividing luciferase activity of reporter alone by luciferase activity of reporter in the presence of Hr, TSA, or both. Experiments were done in duplicate and repeated at least five times with similar results. MLV tk-luc, MLVx2 tk-luc, pRK5myc-

rhr, and pRK5myc-rhr 1–568 have been described elsewhere (17). Reporter genes with the MBP and Pcp-2 TREs (28, 29) were generated by synthesis and annealing of the indicated oligonucleotides and subcloning into tk-luc. Constructs were sequenced to verify sequence and orientation of inserted fragments. MBP TRE: 5′-AGCTATGGGACCTCGGCTGAGGACACG GCTCGAG3′,5′AGCTCTCGAGCCGTGTCCTCAGCCGAGGTC CCAT-3′; Pcp-2 TRE: 5′ AGCTAGGCCTTCTCAGGTCAGAGA CCAGGAGACTCGAG-3′, 5′-AGCTCTCGAGTCTCCTGGTCTC TGACCTGAGAAGGCCT-3′.

CoIP Assay

Nuclear extracts were prepared from p21 rat cerebellum as described (77) except that extracts were diluted 1:3 in water before storage at −80 C. Protein concentration was determined by Bradford assay (Bio-Rad Laboratories, Inc.). For each immunoprecipitation reaction, 400 μg of cerebellar nuclear extract were incubated with antiserum overnight at 4 C. Protein A-Sepharose beads (Sigma) were added for 2 h at 4 C. Immunoprecipitates were collected by centrifugation and washed three times in IP buffer (1% Nonidet P-40; 10% glycerol; 150 mM NaCl; 50 mM Tris, pH 7.5). Proteins were separated by SDS-PAGE and Hr was detected by Western analysis with Hr antiserum. HDAC1-, HDAC2-, and HDAC3-specific antisera were obtained from Sigma, and TR-specific antisera were purchased from Affinity BioReagents, Inc. (Golden, CO) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

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