

Genome-wide coexpression of steroid receptors in the mouse brain: Identifying signaling pathways and functionally coordinated regions

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Steroid receptors are pleiotropic transcription factors that coordinate adaptation to different physiological states. An important target organ is the brain, but even though their effects are well studied in specific regions, brain-wide steroid receptor targets and mediators remain largely unknown due to the complexity of the brain. Here, we tested the idea that novel aspects of steroid action can be identified through spatial correlation of steroid receptors with genome-wide mRNA expression across different regions in the mouse brain. First, we observed significant coexpression of six nuclear receptors (NRs) [androgen receptor (*Ar*), estrogen receptor alpha (*Esr1*), estrogen receptor beta (*Esr2*), glucocorticoid receptor (*Gr*), mineralocorticoid receptor (*Mr*), and progesterone receptor (*Pgr*)] with sets of steroid target genes that were identified in single brain regions. These coexpression relationships were also present in distinct other brain regions, suggestive of as yet unidentified coordinate regulation of brain regions by, for example, glucocorticoids and estrogens. Second, coexpression of a set of 62 known NR coregulators and the six steroid receptors in 12 non-overlapping mouse brain regions revealed selective downstream pathways, such as *Pak6* as a mediator for the effects of *Ar* and *Gr* on dopaminergic transmission. Third, *Magel2* and *Irs4* were identified and validated as strongly responsive targets to the estrogen diethylstilbestrol in the mouse hypothalamus. The brain- and genome-wide correlations of mRNA expression levels of six steroid receptors that we provide constitute a rich resource for further predictions and understanding of brain modulation by steroid hormones.

neuroendocrinology | nuclear receptors | transcription regulation | estrogens | glucocorticoids

Steroid receptors are part of the superfamily of nuclear receptors (NRs) that act as transcription factors regulating expression of numerous biologically important target genes (1). Their transcriptional activity is induced by steroid hormones, which respond to changed demands in terms of reproductive status, mineral balance, or stressful physical and psychological challenges. A crucial site of action is the brain, where these hormones have strong modulatory effects on physiological regulation, cognitive function, mood, and behavior. They do so by changing cellular responsiveness to a variety of neurotransmitters and peptides, and by inducing morphological changes (2, 3). Understanding the effects of steroid hormones on the brain faces the challenge to identify in as many as 900 different brain nuclei (4) both the highly cell-specific target genes that mediate the hormone effects (5, 6) and the signaling factors that mediate or influence steroid receptor signaling. The latter include proteins affecting prereceptor metabolism, interacting transcription factors (7), and downstream NR coregulator proteins (1). Even if the effects of steroid hormones are well-studied in

specific regions (1, 8), overall, the brain steroid receptor targets and mediators remain largely unknown.

In situ hybridization (ISH) has been used to identify the functional roles of the 49 NR genes in adult mouse brain based on the clustering of the NR expression patterns in anatomical and regulatory networks (9). In this study, we substantially extended this approach to identify targets and signaling partners of the steroid receptors, and relationships between different regions of the mouse brain, based on genome-wide coexpression with steroid receptors. The Allen Brain Atlas (ABA) (4) is the most comprehensive repository of ISH-based gene expression in the adult mouse brain. We used the ABA to identify genes that have 3D spatial gene expression profiles similar to steroid receptors.

To validate the functional relevance of this approach, we analyzed the coexpression relationship of the glucocorticoid receptor (*Gr*) and estrogen receptor alpha (*Esr1*) and their known transcriptional targets in specific brain regions. We then exploited these associations to derive hypotheses about the functional role of receptors in brain regions with no previously known effects of steroids. Furthermore, we studied the region-specific coexpression of NRs and their downstream mediators (coregulators) to identify specific partners mediating the hormonal effects on dopaminergic transmission. Finally, to illustrate the potential of using spatial coexpression to predict region-specific steroid receptor targets

Significance

Steroid hormones coordinate the activity of many brain regions by binding to nuclear receptors that act as transcription factors. This study uses genome-wide correlation of gene expression in the mouse brain to discover (i) brain regions that respond in a similar manner to particular steroids, (ii) signaling pathways that are used in a steroid receptor and brain region-specific manner, and (iii) potential target genes and relationships between groups of target genes. The data constitute a rich repository for the research community to support further new insights in neuroendocrine relationships and to develop novel ways to manipulate brain activity in research or clinical settings.

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in the brain, we identified and validated genes that responded to changes in estrogen in the mouse hypothalamus (HY).

Results

Spatial Expression Reveals Known Sites of Action of Steroid Receptors in the Mouse Brain.

We first analyzed the mRNA expression of six nuclear steroid receptors [*Esr1* and estrogen receptor beta (*Esr2*), androgen receptor (*Ar*), progesterone receptor (*Pgr*), *Gr*, and mineralocorticoid receptor (*Mr*)] across the brain using the 3D spatial gene expression data from the ABA (4). We generated a general overview of the expression of each receptor across 12 nonoverlapping brain structures covering the entire brain: isocortex; olfactory areas (OLF), hippocampal formation, cortical subplate, striatum (STR), pallidum (PAL), cerebellum, thalamus (TH), HY, midbrain (MB), pons, and medulla (Fig. 1A). The expression profiles generally correspond to the known distribution and sites of action of different receptors (9), and provide comprehensive information at the higher aggregation level of brain regions described here. For example, *Esr1* is highly expressed in the HY, OLF, and cortical subplate. Within the HY, *Esr1* shows high expression in the arcuate hypothalamic nucleus (ARH), and medial preoptic nucleus (MPO) (Fig. 1B). *Gr* is highly expressed in the cornu ammonis subdivision 1 (CA1) and dentate gyrus (DG) areas of the hippocampus, cortex, and TH, whereas *Mr* is predominantly expressed in the hippocampus (Fig. 1A). These expression patterns are well in line with the known sites of action of the different receptors across the brain (10, 11).

Genes Spatially Coexpressed with Steroid Receptors Indicate Regional Functional Specificity.

To go beyond the expression profiles of steroid receptors as reported in the literature, we identified genes with similar expression profiles to each of the receptors. Based on the principle of “guilt by association,” these coexpressed genes are likely to be enriched in receptor target genes and receptor signaling partners such as coregulators. For each steroid receptor, we ranked genes based on their spatial coexpression across the whole brain as well as in each of the aforementioned 12 brain structures separately, resulting in 13 ranked lists per receptor (Dataset S1). (Datasets S1–S6 are available at data.3tu.nl/repository/uuid:ecc3b182-d312-4216-9053-a824d0e04d5e.) For each steroid receptor, strongly coexpressed genes within a brain region are likely related to the localized functional role of the receptor. For example, of the top 10 genes coexpressed with *Esr1* across the whole brain, four were previously shown to be regulated by ESR1 and/or estrogens in various tissues (*Gpr101*, *Calcr*, *Ngb*, and *Gpx3*) (12–15). These genes were also coexpressed with *Esr1* in the HY, in line with their functional relationship to *Esr1* in mediating, for example, reproductive and metabolic processes. However, whole-brain correlation of these genes with *Esr1* was also driven by the TH, MB, and PAL, demonstrating less obvious relationships between *Esr1* and these target genes. Strikingly, among the top 10 genes coexpressed with *Gr* across the whole brain, none are strongly coexpressed with *Gr* in the HY, indicating that *Gr* signaling in the HY is rather distinct from *Gr* signaling in the cortex, STR, TH, and MB.

In addition, we analyzed the functional enrichment of genes coexpressed with *Gr* and *Esr1* in the 12 brain regions (Table S1). *Esr1*-coexpressed genes were enriched for neuropeptide regulation in the HY as well as the cerebellum. A number of *Gr*-associated genes in the HY were related to glia and oligodendrocyte development, supporting the known effects of *Gr* on these processes in the HY (16).

Glucocorticoid-Responsive Genes Are Highly Expressed with *Gr* in HP, Pons, MB, and Whole Brain.

To test the validity of our hypothesis that coexpressed genes constitute candidate targets of steroid receptors, we assessed the extent of coexpression between *Gr* and known GR target genes. Because *Gr* has an important role in mediating transcription of genes involved in coping with stress within the hippocampus (2), we analyzed the coexpression of glucocorticoid (GC)-responsive genes (i.e., likely GR targets) with *Gr* in the whole brain and hippocampus, and in its

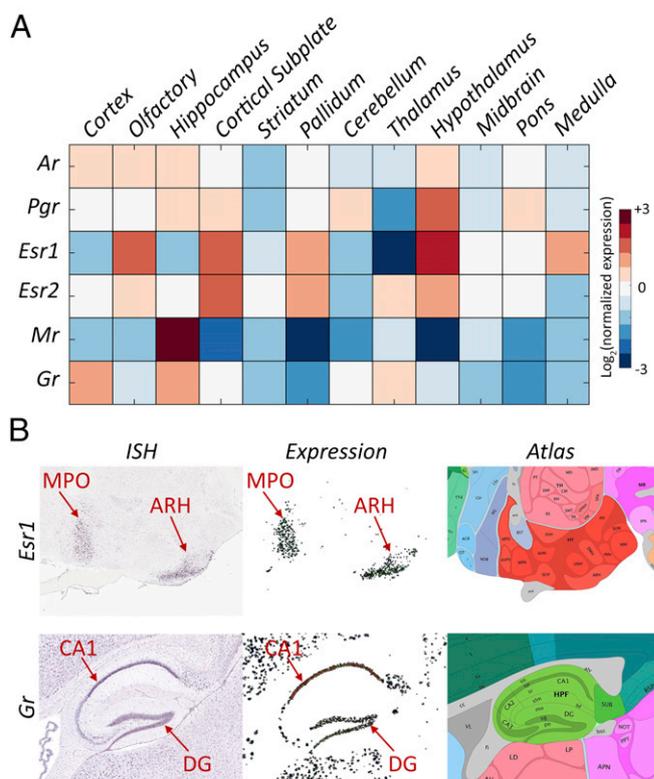


Fig. 1. Expression of steroid receptors in the mouse brain. (A) Expression of six steroid receptors across the 12 brain regions. Reported values are the average expression energy per region normalized to the average expression across the whole brain and then \log_2 -transformed. (B) Example sagittal sections from the Allen Brain Atlas (4) showing the ISH (Left), expression mask (Middle), and corresponding atlas section (Right) of *Esr1* in the HY (Top) and *Gr* in the hippocampus (Bottom). Red arrows indicate the MPO, ARH, CA1, and DG.

substructures, such as the DG and the different subregions of the CA (Fig. 2A and Dataset S2). The set of GC-responsive genes we considered originates from experiments where male rats were exposed to GC treatment in a chronic restraint stress (CRS) condition as well as in a control situation (17). These experiments resulted in three sets of genes differentially expressed in DG neurons: (i) GC-responsive genes in CRS rats, (ii) GC-responsive genes in control rats, and (iii) genes that show differential expression in GC treatment for both conditions (common GC-responsive genes).

As expected, GC-responsive genes are significantly coexpressed with *Gr* in the DG (where they were identified) but, interestingly, also in the whole brain and in the CA3 region [false discovery rate (FDR)-corrected $P < 1.8 \times 10^{-3}$; Mann–Whitney U test]. The significant coexpression of GC-responsive genes in the CA3 area indicates that those cells in CA3 that do express *Gr* (10) may be functionally linked to DG granule cells in terms of their response to GCs. Of note, only those genes that responded to GC treatment in stressed and control rats (common GC-responsive genes) showed a significant coexpression with *Gr* in the DG, CA1, and (very substantially) CA3 regions of the hippocampus. The data reveal that only the subset of invariant, context-independent GR target genes is related to constitutive coexpression with *Gr*, even if the correlation data come from “control” conditions.

The coexpression of the GC-responsive gene sets with *Gr* was not significant for areas such as the HY and the cortex. We initially considered these negative control regions, given that the target genes were identified in microdissected DG granule neurons (17) and the presumed high degree of cell specificity. However, the coexpression of *Gr* with GC-responsive genes in CA3 prompted us to test whether this coexpression also occurs in other brain areas. Fig. 2B shows that the set of common GC-responsive genes is not only coexpressed with

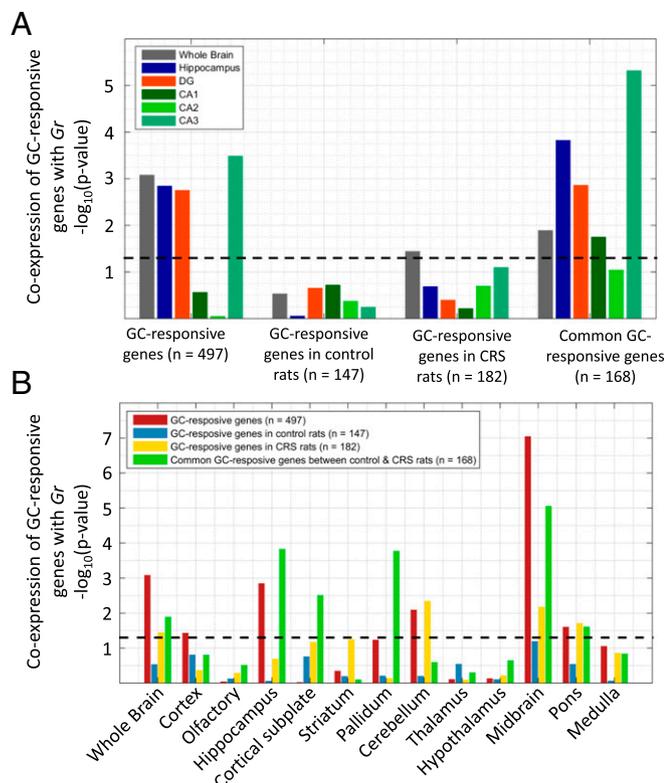


Fig. 2. Coexpression of GC-responsive genes and *Gr* in the hippocampus. (A) Coexpression of four GC-responsive gene sets with *Gr* in the whole brain, hippocampus, DG, CA1, CA2, and CA3. (B) Coexpression of four GC-responsive gene sets with *Gr* across the whole brain, as well as the 12 major brain structures. All bars indicate the $-\log_{10}$ of the Wilcoxon rank sum test, and the dashed line indicates the significance level at $P = 0.05$.

Gr in the hippocampus (DG, CA1, and CA3) but also in the cortical subplate, PAL, and MB. These associations indicate a potential, as yet unknown, relationship between these three brain areas in terms of endocrine regulation, in accordance with the notion that the cellular responses to GCs can be similar in distributed parts of brain networks (18). Taken together, these results show that GR targets are coexpressed with *Gr* in the DG, the region where responsiveness was measured, as well as pointing to other brain regions that might share the same regulation mechanism.

Sexually Dimorphic Genes Are Highly Coexpressed with *Esr1* in the HY.

To illustrate the generalizability of our approach to other receptors and brain regions, we followed the same approach to analyze the coexpression of *Esr1* and its putative targets. Xu et al. (19) showed that a set of 16 genes, including *Esr1*, has sexual dimorphic expression in the adult mouse HY. In addition, they showed that these 16 genes are sensitive to gonadal steroids (also in the male mouse brain) and that some are necessary for effects of estrogens on sexually dimorphic behavior (19), making this set of ESR1 targets in the HY quite valuable.

Table S2 shows the correlation values for each of the 15 sexually dimorphic genes with *Esr1* in whole brain, as well as in the HY, based on data from the ABA. The set of 15 genes is significantly correlated to *Esr1* based on whole-brain analysis (FDR-corrected $P = 8.69 \times 10^{-14}$, Mann-Whitney U test) as well as the hypothalamic expression pattern ($P = 3.85 \times 10^{-10}$). To test whether the correlation between the 15 genes and *Esr1* is HY-specific, we repeated the analysis for all 12 brain structures. Fig. 3A shows that sexually dimorphic genes are mostly correlated to *Esr1* in the HY, PAL, TH, and STR ($P < 10^{-6}$). Similar to the results obtained for GR target genes, we observed high coexpression outside the main region of action (e.g., in the PAL), suggesting that these brain regions share aspects of their

transcriptional response to estrogen receptor activation. Furthermore, sex steroid receptors (*Esr1*, *Esr2*, and *Pgr*) showed higher coexpression levels with the sexually dimorphic genes with respect to the stress steroid-related *Mr* and *Gr* in the HY (Fig. 3B). The strongest coexpression was with *Esr1*, indicating that the hypothalamic sexual dimorphism genes are mainly, but probably not exclusively, related to *Esr1*. Taken together, these results show that spatial coexpression can pinpoint context-specific actions of steroid receptors (in this case, *Gr* and *Esr1*) and yields region-specific coexpressed genes, a very rich resource with which to generate hypotheses about steroid receptor targets.

Region-Specific Coregulator Analysis Points to Dopaminergic Transmission via *Pak6*.

So far, we have analyzed the potential of genes coexpressed with receptors to include region-specific targets. However, because correlation only indicates association rather than causation, coexpressed genes can also include coregulators of steroid receptors. Previous studies have shown the signaling pathways of steroid receptors to differ across brain regions in a gene-specific manner (1, 20). To identify putative region-dependent coregulators of steroid receptors, we analyzed the coexpression relationships of each steroid receptor and a set of 62 NR coregulators as present on a peptide array (21) (complete data are provided in Dataset S3). Fig. 4A shows that the expression of coregulators varies greatly across the different brain regions. For example, although *Ncoa1* is expressed in a fairly homogeneous manner, conforming to earlier results (20), *Ncoa4* is substantially enriched in the caudal brain regions.

The coexpressions of coregulators with the *Ar*, *Gr*, and *Mr* differ greatly across different brain regions, indicating selective coregulation (Fig. 4B–D). For example, the AR/GR coactivators *Pias2* (22) and *Ncoa4* (23) are highly coexpressed with *Gr* in the MB and HY, respectively (Fig. 4C). However, both coactivators are not coexpressed with *Ar* within the same regions even though the relative abundance of *Ar* in the MB and the HY is higher than *Gr* (Fig. 1A). *Mr* is predominantly expressed in the

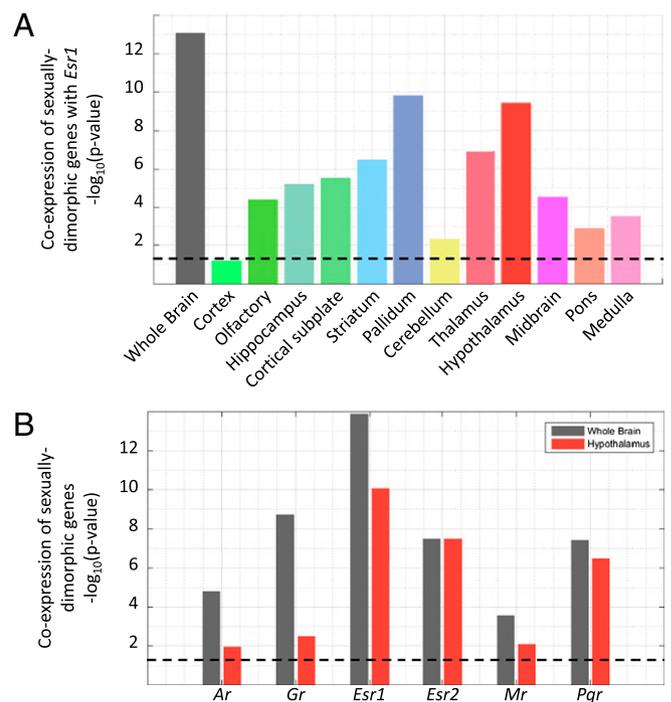


Fig. 3. Coexpression of sexually dimorphic genes and *Esr1* in the HY. (A) Coexpression of 15 sexually dimorphic genes with *Esr1* across the mouse brain. (B) Coexpression of the 15 sexually dimorphic genes with the six steroid receptors across the whole brain, as well as the HY. All bars indicate the $-\log_{10}$ of the Wilcoxon rank sum test, and the dashed line indicates the significance level at $P = 0.05$.

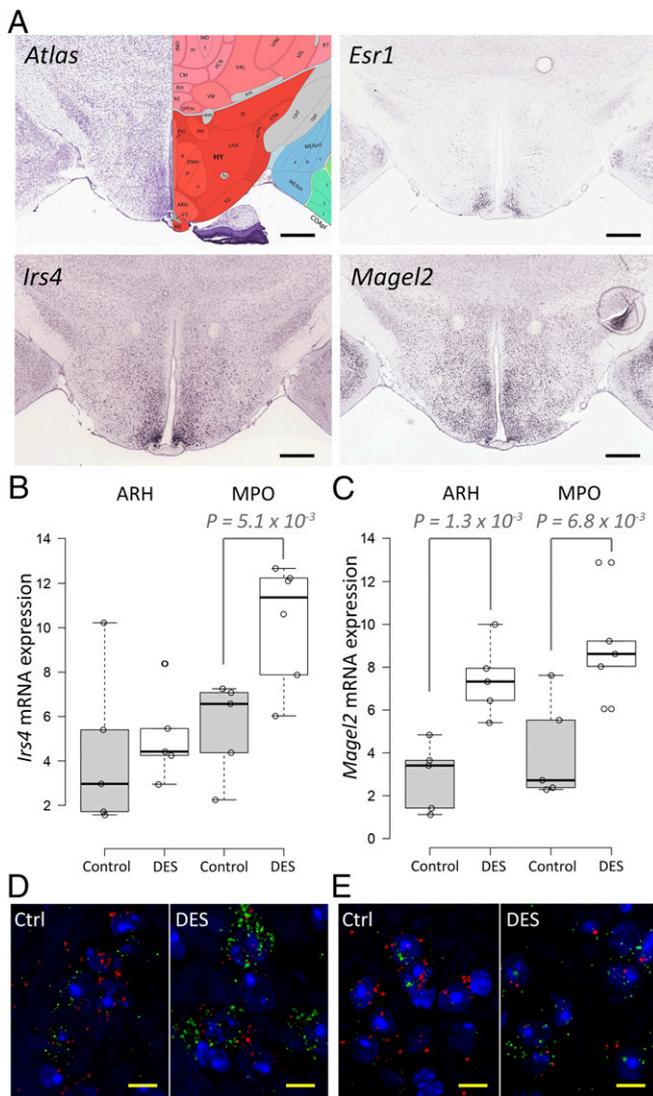


Fig. 5. Highly coexpressed genes are potential steroid targets. (A) Coronal ISH sections showing the expression of *Esr1*, *Irs4*, and *Magel2*. (Scale bars, 600 μm .) Data taken from the Allen Brain Atlas (4). Response of *Irs4* (B) and *Magel2* (C) to DES treatment in castrated mice in the MPO and ARH using dISH. (D) dISH of *Esr1* (red) and *Irs4* (green) in the anterior HY. (Scale bars, 10 μm . Magnification, 100 \times .) (E) dISH of *Esr1* (red) and *Magel2* (green) in the anterior HY. (Scale bars, 10 μm . Magnification, 100 \times .) mRNA expression in ISH was quantified as the percentage of the image surface with positive signal. Reported *P* values are calculated with a one-sided, two-sample *t* test with a significant level at $P < 0.05$.

Discussion

Because nuclear steroid receptors act as transcription factors, they may be expected a priori to coexpress with their target genes and signaling partners. In the brain, the effects of steroid receptors are region-specific, and by analyzing their spatial coexpression relationships across different brain regions, we can define potential targets and partners, as well as parallels between brain areas. The complexity and large variability in gene expression across the brain have forced many studies to analyze either brain-wide expression of a small set of genes or genome-wide expression in a few regions. The availability of high-resolution ISH-based expression maps of the mouse brain in the ABA allows the identification of all genes with a similar expression pattern across many brain regions that might indicate functional similarity between the gene products (29). We provide a comprehensive description of the coexpression of genes with six receptors of gonadal and adrenal steroid hormones in the male mouse brain. Our

results demonstrate that genes that are spatially coexpressed with receptors in a region-specific manner can enhance our understanding of brain modulation by steroid hormones.

Using genome-wide spatial coexpression analysis, we observed strong coexpression of known GR transcriptional targets in the hippocampus and known ESR1 transcriptional targets in the HY. These observations support our hypothesis that genes showing strong coexpression with a steroid receptor are enriched in transcriptional targets and/or coregulators of that receptor. In addition, the unanticipated coexpression of genes with these receptors outside their known sites of action may extend our understanding of the coordinated steroid response of the brain. For example, the high coexpression between *Gr* and its GC-responsive target genes (originally derived from the DG) in CA3, the MB, and the PAL is in line with a network that has been referred to as the neuro-circuitry of stress (30). Likewise, dendritic complexity of neurons and excitability are modulated by GCs and stress across different brain regions simultaneously (18). Such similar responses of distinct brain regions suggest similar cellular machinery, and thus similarly correlated gene expression with the responsible receptor.

For the genes that are expressed in a sex-specific manner, we confirmed their coexpression with *Esr1*, *Esr2*, and *Pgr*, which is in accordance with their regulation by gonadal steroids (19). Lack of coexpression with *Ar* may reflect the fact that many testosterone effects on the HY are mediated by estrogen receptors after aromatization of testosterone into estradiol. It is as yet unclear whether the significant coexpression with *Pgr* reflects simply coexpression of *Esr1* and *Pgr* or also points to progesterone regulation of these genes. Regardless, we extended the coexpression between sexually dimorphic genes to extrahypothalamic sites, pointing to a parallel regulation in, at least, the PAL, a region that includes the bed nucleus of the stria terminalis, where regulation by (nonspecified) gonadal hormones has been observed (19).

Our analysis of the coexpression of coregulators and steroid receptors identified known relationships, such as the high coexpression between *Ncoa1* and *Mr* in the hippocampus (20). More importantly, this brain-wide analysis provides an overview of potentially unknown relationships between steroid receptors and coregulators. By focusing on dopaminergic regions (VTA and SN), we identified strong coexpression of *Pak6* with *Ar* as well as *Gr*. Of interest, *Pak6* is a known AR coregulator (31) and *Pak6* KO mice show several locomotion and behavioral deficits that are likely related to disturbed dopaminergic transmission (32). Thus, this example of *Pak6* coexpression underscores the feasibility of our methodology to find potential partners of nuclear steroid receptors. Of note, steroid receptor/coactivator interactions may be induced with a certain degree of specificity by selective modulator types of steroid receptor ligands (24, 33). The coexpression of steroid receptors with their coactivators may not only predict steroid responsiveness but also point to selective activation of particular circuits with synthetic ligands (24).

To test whether spatial coexpression can be used to predict transcriptional targets of steroid receptors in the brain, we used qPCR and dISH to assess if genes strongly coexpressed with *Esr1* in the HY include any ESR1 targets. Among the tested genes we identified two estrogen-regulated genes: *Irs4*, a previously known ESR1 target (19); and *Magel2*, a previously unidentified ESR1 target. Loss of *Magel2* leads to impaired reproduction, providing an immediate link to estrogen regulation (34). This gene is deleted in Prader-Willi syndrome, which is associated with hypogonadotropic hypogonadism, obesity, and hyperphagia (35). Likewise, *Irs4* has a role in hypothalamic leptin signaling and regulation of metabolism (36). Therefore, hypothalamic estrogen responsiveness of *Magel2* and *Irs4* may be related to estrogen effects on metabolism (37). The presently modest predictive power may be improved by incorporating the effect size (i.e., the absolute expression of a gene), given the values for true positives *Irs4* and *Magel2*. Also, the presence of conserved steroid response elements on the DNA could be a useful additional filter (38).

The enrichment of known targets and coregulators of a certain NR within the same brain regions where the NR is

expressed confirms the validity of our analysis. Our approach is even strengthened by the notion that the receptor and its targets and/or coregulators are significantly coexpressed despite the genome-wide and brain-wide qualitative approach of measuring mRNA levels using ISH. However, there are some intrinsic limitations to the analysis. First, although the quality of ISH is overall high, it is insufficient for some genes. Of the three datasets covering expression of *Gr*, only one was of sufficient quality. Also, *Ncoa1*, which codes for an important coregulator for ESR1 and GR (1, 20), is expressed at low levels and not significantly associated with the two receptors. Consequently, there is the risk for increased false-negative results associated with a genome-wide approach using these data. Second, the ABA maps the expression of all genes under the same normal conditions. This dataset, although unique in its brain-wide and genome-wide coverage, does not include variations between individuals or context-specific expression patterns (*SI Materials and Methods*).

Our approach relies on Pearson's correlation as a measure of similarity between 3D expression patterns of genes, summarized to 200- μ m isotropic voxels. Although using the expression volumes instead of the original ISH slices simplifies computations and reduces noise effects, the lower resolution yields the analysis of small brain nuclei unreliable. For example, the very small number of voxels representing dorsal raphe nucleus in the 3D atlas hampered analysis of the serotonergic dorsal raphe nucleus. By using

correlation as a measure of coexpression, we detect both direct and indirect statistical associations between genes rather than causal relationships, yielding functional validation using expression measurements (qPCR and/or dISH) and ChIP analysis crucial to confirm predicted associations as well as causality.

Concluding, we have shown that nuclear steroid hormone receptors coexpress with genes not known to associate, and in brain regions where steroids were not known to be active. These findings point toward the brain region-specific signaling machinery of the steroid receptors.

Materials and Methods

SI Materials and Methods includes detailed descriptions of the ABA mouse data, data preprocessing steps, spatial coexpression, gene set analysis, selecting targets for experimental validation, rank sum analysis, qPCR validation, dISH, and the ChIP-Seq experiments.

All animal experiments were performed with the approval of the Animal Ethics Committee at Erasmus Medical Center.

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