Multiple ERβ antisera label in ERβ knockout and null mouse tissues

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ABSTRACT

In the process of characterizing a custom-made affinity-purified antiserum for estrogen receptor beta (ERβ), ck5912, we used a number of common tests for specificity of ck5912 along with that of 8 commercially available ERβ antisera: Affinity Bioreagents PA1-310B, Invitrogen D7N, Upstate 06-629, Santa Cruz H150, Y19, L20, 1531, and Abcam 9.88. We tested their recognition of recombinant ERβ (rERβ) versus rERα, ERβ versus ERα transfected into cell lines, as well as labeling in wildtype (WT) versus estrogen receptor beta knockout (βERKO) and null (ERβ$_{ST}$$^{L-/L-}$) mouse ovary, hypothalamus, and hippocampus. To our surprise, we found that while most of these antisera passed some tests, giving the initial impression of specificity, western blot analysis showed that all of them recognized apparently identical protein bands in WT, βERKO and ERβ$_{ST}$$^{L-/L-}$ tissues. We share these results with the goal of helping other researchers avoid pitfalls in interpretation that could come from use of these ERβ antisera.

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1. Introduction

The discovery of estrogen receptor β (ERβ; Kuiper et al., 1996) opened a new door to understanding physiological actions of estradiol. Neuroscientists who study hormone effects in the brain are particularly interested in ERβ because pharmacological and knockout studies point to ERβ as being important in learning and memory (Liu et al., 2008), anxiety (Imwalle et al., 2005; Tomihara et al., 2009), and aggression (Ogawa et al., 1999).

First cloned in rat and subsequently in human and mouse, the ERβ gene contains eight exons and shares a high degree of sequence homology with estrogen receptor alpha (ERα) in the DNA and ligand binding domains (Kuiper et al., 1996; Mosselman et al., 1996; Tremblay et al., 1997). Additionally, the ERβ gene undergoes alternative splicing leading to the expression of several isoforms. One splice variant, ERβ2, contains a 54 bp insert leading to an additional 18 amino acids; other splice variants, called delta variants, lack entire exons (Chu and Fuller, 1997; Lu et al., 1998). Like ERα, ERβ is traditionally thought of as a transcription regulator. However, estradiol also has many rapid effects on neurons that likely involve ER signaling outside of the nucleus and some pharmacological evidence implicates ERβ in these rapid, extranuclear effects (Zhao and Brinton, 2007; Kramár et al., 2009).

Understanding the function of ERβ requires knowing where it is located and what proteins it interacts with, which in turn, requires reliable and specific antibodies. Early studies with ERβ antisera showed some agreement but also some discrepancies between localization of ERβ immunoreactivity (Li et al., 1997; Shughrue and Merchenthaler, 2001) and mRNA (Shughrue et al., 1997), raising concerns about ERβ antisera (Warner et al., 2003; Shughrue and Merchenthaler, 2001). Then, in 2001, the Z8P ERβ antiserum showed consensus between ERβ mRNA and protein expression in many brain areas, including areas that previously were controversial (Shughrue and Merchenthaler, 2001). Unfortunately, however, Z8P is no longer available.

Our lab is particularly interested in ERβ function in the hippocampus, including its colocalization with other proteins. To facilitate studies of ERβ, we produced two ERβ antisera raised in chicken for use in conjunction with other commercially available antisera. The more promising of these was ck5912. In the process of characterizing ck5912, we performed a number of commonly used tests for its specificity, along with the specificity of 8 commercially available ERβ antisera. To our surprise, we found that while some of these antisera passed many tests, some did not. Most significantly, we found that all the ERβ antisera we tested detected immunoreactivity in tissues from two independently generated strains of ERβ knockout mice, the βERKO mouse (Krege et al., 1998) and Chambon's recently generated ERβ null mouse (ERβ$_{ST}$$^{L-/L-}$; Antal et al., 2008). We share these findings with the goal of helping other researchers avoid pitfalls in interpretation that could arise from the use of these ERβ antisera.

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Table 1
Summary of ERβ antisera tested for specificity.

Antisera	Company, catalog number	Antigen; dilution used	Host species
PA1-310B	Thermo Scientific (formerly Affinity Bioreagents), PA1-310B	Synthetic peptide corresponding to residues C(467) SSTEDSKNKESSQNLQSQ (485) of rat ERβ; 1:1000	Rabbit
ck5912	Custom	Synthetic peptide corresponding to residues C(467) SSTEDSKNKESSQNLQSQ(485) of rat ERβ; 1:10,000	Chicken
D7N	Invitrogen (formerly Zymed), 51-7700	19 amino acid synthetic peptide derived from the C-terminus of human ERβ; 1:250	Rabbit
H150	Santa Cruz, sc-8974	Amino acids 1–150 of human ERβ; 1:1000	Rabbit
06-629	Millipore (formerly Upstate), 06-629	Peptide (YAEPQKSPWCEARSLEHT) representing amino acids 54–71 of rat and mouse ERβ and amino acids 46–63 of human ERβ; 1:1000	Rabbit
1531	Santa Cruz, sc-53494	Amino acids 256–505 of human ERβ; 1:250	Mouse
Y19	Santa Cruz, sc-6821	N-terminus of mouse ERβ; 1:250	Goat
L20	Santa Cruz, sc-6822	C-terminus of human ERβ; 1:250	Goat
9.88	Abcam, ab16813	Recombinant full-length human ERβ; 1:1000	Mouse

2. Materials and methods

2.1. Animals

All animal procedures were performed in accordance with the *National Institute of Health Guide for the Care and Use of Laboratory Animals* and were approved by the Northwestern University Institutional Animal Care and Use Committee. βERKO and C57/BL6J breeder mice were purchased from Jackson labs. Mice used for experiments were obtained by in-house breeding with genotype confirmation by Transnetyx. Tissues from $ERβ_{ST}^{L−/L−}$ and wild-type mice were a kind gift from Dr. Shaila Mani (Baylor College of Medicine, Houston, TX).

2.2. Antisera

For ERβ: antisera are summarized in Table 1. For GFP: Clontech JL8 mouse anti-GFP 632380 (1:1000).

2.3. Western blots

Mice were deeply anesthetized with sodium pentobarbital (80 mg/kg, i.p.) and perfused with ice-cold RIPA buffer lacking detergents (in mM: 50 Tris–HCl, 150 sodium chloride, 1 EDTA, 1 sodium orthovanadate, 0.1 phenylmethylsulfonyl fluoride, 50 sodium fluoride, 10 sodium pyrophosphate, 20 glycerophosphate, with 1 μg/ml leupeptin and 1 μg/ml aprotinin). Brains and ovaries were rapidly removed and placed on ice. Ovaries, hypothalamus, and hippocampi were dissected and homogenized in RIPA buffer containing 1% nonidet P40, 0.25% sodium deoxycholate, and 0.1% sodium dodecyl sulfate, incubated on ice for 25 min, and spun at $1000 \times g$ for 10 min to remove large cell fragments and nuclear material. The supernatant was kept as the whole cell fraction. The protein sample was mixed with Laemmli sample buffer (62.5 mM Tris–HCl, pH 6.8, 25% glycerol, 2% SDS, 0.01% Bromophenol blue, 5% β-mercaptoethanol), boiled for 5 min, and separated on a 10% SDS-PAGE gel. After electrophoresis, proteins were transferred to polyvinylidene difluoride membranes (Millipore). The membrane was blocked in 5% nonfat milk and probed with primary antiserum (see Table 1). For preadsorption experiments, diluted antiserum was incubated with 1000 fold excess antigenic peptide and kept at 4 °C overnight before probing membranes. Blots were then incubated with horseradish peroxidase coupled anti-rabbit, anti-goat, or anti-mouse IgG secondary antibody (Vector Laboratories) and proteins were visualized using enhanced chemiluminescence (ECL Plus, Amersham Biosciences). Recombinant estrogen receptor alpha (rERα) and recombinant estrogen receptor beta (rERβ) protein were purchased from Invitrogen.

2.4. Ovary immunohistochemistry

Mice were deeply anesthetized with sodium pentobarbital (80 mg/kg, i.p.) and perfused with 4% paraformaldehyde/3.75% acrolein in phosphate buffer. Ovaries were removed, postfixed for 1 h in paraformaldehyde, cryoprotected, and sectioned. Immunostaining was performed using a standard avidin–biotin peroxidase method as previously described with slight modifications (Rudick and Woolley, 2003). Tissue was incubated in primary antiserum overnight and in secondary antiserum for 1 h. Sections were counterstained with hematoxylin and coverslipped.

2.5. Cell culture, transfection and immunostaining

HT22 cells were a kind gift from Dr. Pamela Maher (The Scripps Research Institute, La Jolla, CA). Cells were grown on 100 mm tissue culture dishes or glass coverslips and maintained in DMEM media supplemented with 10% fetal calf serum and 1% Pen-Strep (Invitrogen) at 37 °C in a 10% CO_2 atmosphere. Cell density was maintained at ≤70% confluence and cells were split using 0.05% trypsin/0.53 mM EDTA (Invitrogen). HT22 cells were transiently transfected with plasmid expression vectors containing inserts for GFP, ERα-GFP, or ERβ2-GFP (kind gift of Dr. Toni Pak, Loyola University Chicago Stritch School of Medicine, Maywood, IL) using Lipofectamine 2000 according to the manufacturer's instructions. Twenty-four hours after transfection, cells were fixed for immunocytochemistry or collected for western blot. For western blots, cells were scraped into RIPA buffer containing 1% nonidet P40, 0.25% sodium deoxycholate, and 0.1% sodium dodecyl sulfate, and spun to obtain whole cell fractions as above. For immunocytochemistry, cells were fixed for 15 min in methanol at −20 °C. After rinsing with phosphate buffered saline (PBS), cells were incubated for 1 h in 3% goat serum, 10% BSA, and 0.3% DMSO in PBS to block nonspecific staining. Cells were then incubated overnight with primary antiserum in 1% goat serum, 2% BSA, and 0.3% DMSO in PBS. Cells were rinsed and incubated with chicken or rabbit IgG coupled to Alexa Fluor 568 for ERβ or mouse IgG coupled to Alexa Fluor 488 for GFP. Cells were coverslipped and then imaged using a PerkinElmer Ultraview spinning disc laser confocal microscope. Experiments were also done using human embryonic kidney (HEK) 293 cells. Methods were as above except that cells were maintained in 5% CO_2 and split using mechanical dissociation.

2.6. Reverse transcriptase PCR (rtPCR)

WT and βERKO mice were deeply anesthetized with sodium pentobarbital (80 mg/kg, i.p.). Ovary, hypothalamus, and hippocampus were removed immediately and placed on ice. RNA was extracted from tissue using a Trizol plus purification kit (Invitrogen) according to the manufacturer's instructions. First strand cDNA was generated from 1 μg RNA by reverse transcriptase using the SuperScript III First-Strand Synthesis System (Invitrogen). PCR amplification was performed on 3 μl of cDNA using primers 5′-GCCAATCATCGCTTCTCTAT-3′ and 5′-CCCTCTTTGCTCTTACTGTCCTCT-3′, as described (Krege et al., 1998). ERβ was amplified for 30 or 40 cycles as follows: 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min. Water was used as a negative control. Amplified DNA was run on a 1.75% agarose gel. Hippocampal DNA samples were cut from the gel and sent to ACGT, Inc. for sequencing. Following amplification, hypothalamic DNA samples were purified using a QIAquick PCR purification kit (Qiagen) and sent to ACGT, Inc. for sequencing.
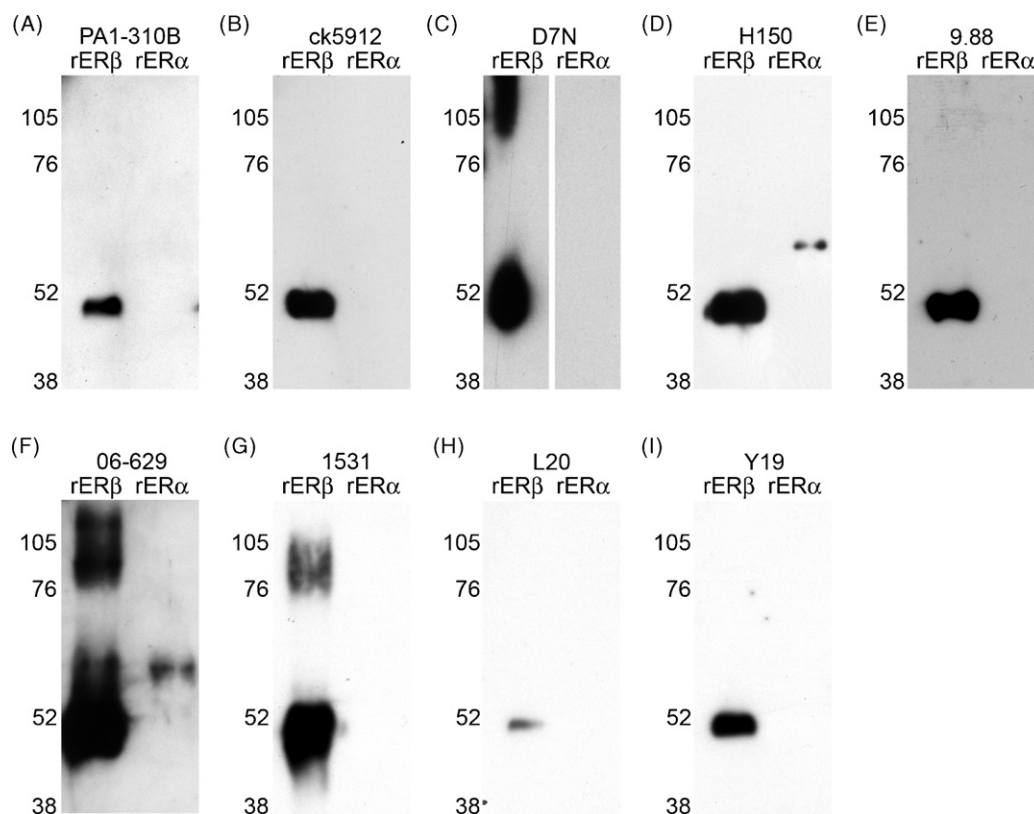
3. Results

Our initial test of ERβ antisera was to confirm specificity for rERβ versus rERα using western blot. Gels were loaded with 0.1 μg rERβ and rERα, and then probed with one of 9 ERβ antisera: PA1-310B, ck5912, D7N, H150, 9.88, 06-629, 1531, L20, or Y19 (Fig. 1A–I). All antisera detected a band of ~50 kDa for rERβ. D7N, 06-629, and 1531 also recognized a band >76 kDa (Fig. 1C, F and G), which could correspond to a cluster of rERβ; however this was not seen with the other antisera. Additionally, both H150 and 06-629 failed this initial test in that they both detected rERα (Fig. 1D and F).
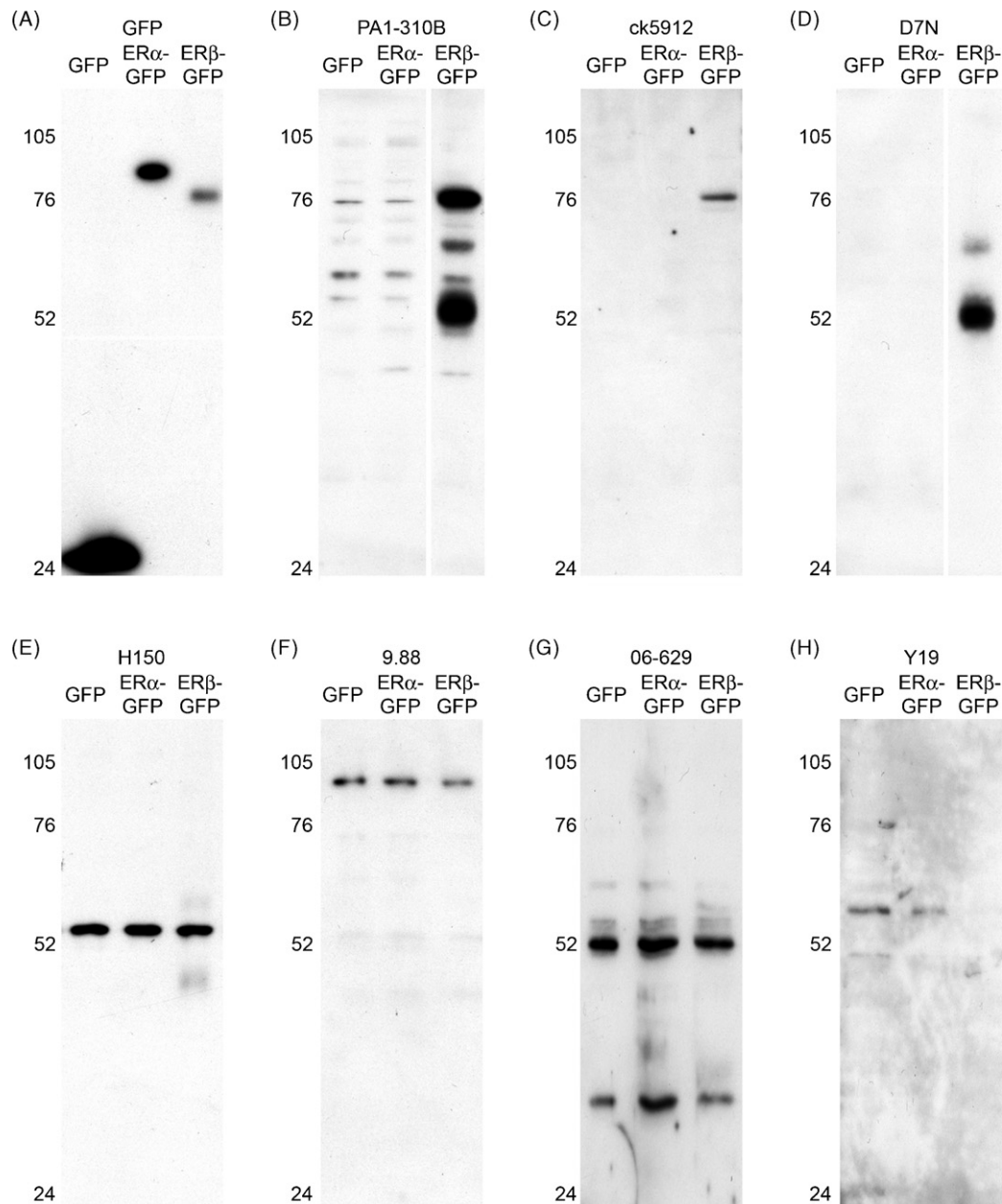
We next tested specificity in cultured cells using western blot and immunocytochemistry. We transfected GFP, ERα-GFP, or ERβ-GFP into the immortalized murine hippocampal cell line, HT22, which is devoid of functional estrogen receptors (Fitzpatrick et al., 2002). Probing western blots with anti-GFP confirmed successful transfection (Fig. 2A). Anti-GFP showed a band at ~27 kDa in GFP-only transfected cells, at ~83 kDa (the predicted molecular weight of GFP-tagged ERα) in ERα-GFP transfected cells, and at ~78 kDa (the predicted molecular weight of GFP-tagged ERβ) in ERβ-GFP transfected cells.

Consistent with previous work (Sheldahl et al., 2008), PA1-310B detected an appropriately sized band of ~78 kDa in ERβ-GFP transfected cells, and also a band at ~52 kDa, which could reflect ERβ cleaved from GFP (Fig. 2B). The upper band was also seen faintly in GFP and ERα-GFP cell extracts. Ck5912 also recognized a band of ~78 kDa and only in ERβ-GFP transfected cells (Fig. 2C), which is the expected result. D7N detected one main band in ERβ-GFP transfected cells, but its molecular weight suggested ERβ cleaved from GFP (Fig. 2D). H150 detected an incorrectly sized single band of ~55 kDa in all transfected cell extracts (Fig. 2E), similar to a previous report (Sheldahl et al., 2008). Abcam 9.88 detected a band of ~100 kDa in all cell extracts (Fig. 2F). 06-629 detected a band of ~52 kDa, as well as a ~38 kDa band in all cell extracts (Fig. 2G). Y19 detected very faint bands only in GFP and ERα-GFP transfected cells (Fig. 2H), and required long exposure times to visualize. Efforts to obtain more definitive results for Y19 by optimizing the protocol were unsuccessful. L20 and 1531 failed to detect any immunoreactivity in transfected cells (not shown). Results were identical when the same experiments were done in HEK 293 cells (not shown).

Because PA1-310B and ck5912 looked the best by western blot and because PA1-310B is a commonly used ERβ antiserum, we next tested both of these antisera with immunocytochemistry in

Fig. 1. Western blots of recombinant ERβ (rERβ) and recombinant ERα (rERα) probed with anti-ERβ antisera. (A) PA1-310B anti-ERβ, (B) ck5912 anti-ERβ, (C) D7N anti-ERβ, (D) H150 anti-ERβ, (E) 9.88 anti-ERβ, (F) 06-629 anti-ERβ, (G) 1531 anti-ERβ, (H) L20 anti-ERβ, and (I) Y19 anti-ERβ. All antisera recognized rERβ with a band of ~50 kDa, as appropriate. D7N, 06-629, and 1531 additionally recognized higher molecular weight bands, which could correspond to a cluster of rERβ. H150 and 06-629 also recognized rERα with a faint band of ~68 kDa.
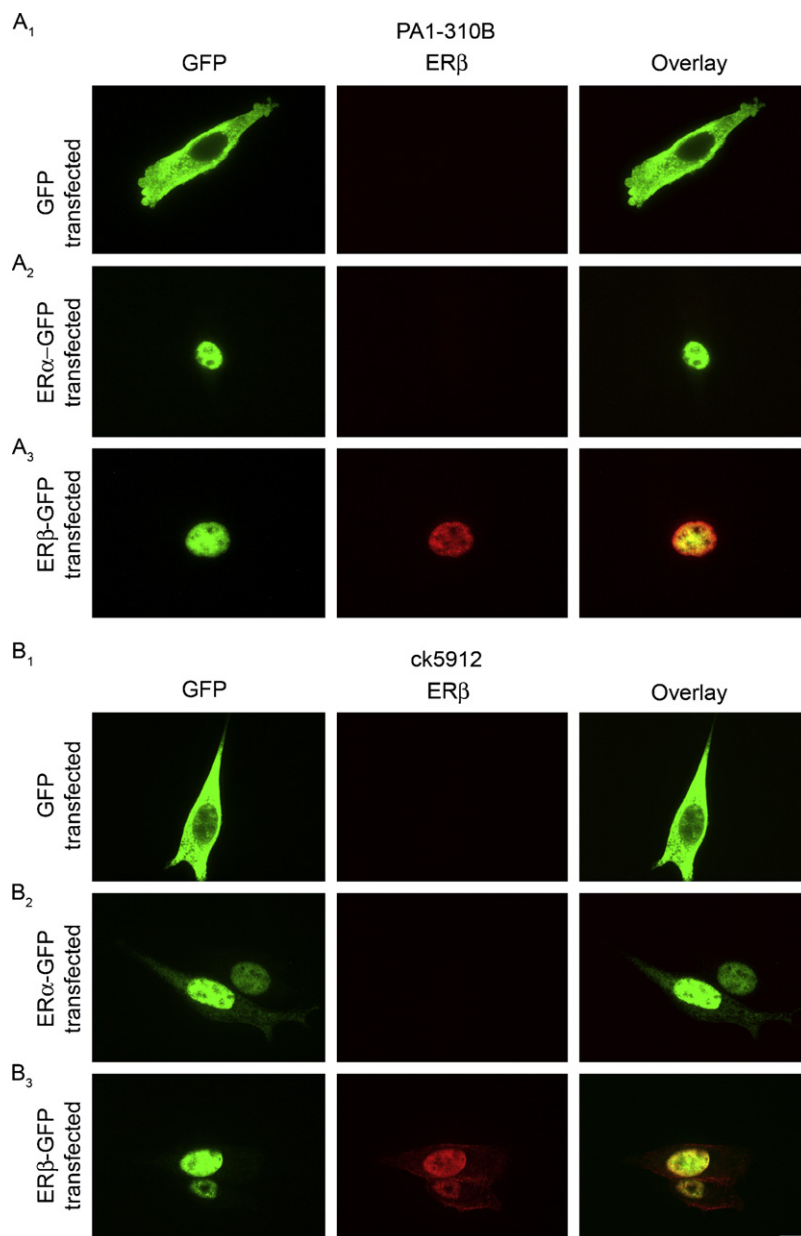
Fig. 2. Detection of ERβ in transfected HT22 cells by western blot. (A–H) Western blots of HT22 cells transfected with GFP, ERα-GFP, or ERβ-GFP and probed with anti-GFP or various anti-ERβ antisera: (A) GFP is detected in all transfected cells, as appropriate. (B) PA1-310B anti-ERβ detected a prominent, appropriately sized band of ∼78 kDa in ERβ-GFP transfected cells, and also a prominent band at ∼52 kDa, which could reflect ERβ cleaved from GFP. PA1-310B also detected faint bands in ERα-GFP transfected cells. (C) ck5912 anti-ERβ detected a single appropriately sized band of ∼78 kDa only in ERβ-GFP transfected cells. (D) D7N anti-ERβ detected a single a band of ∼52 kDa in ERβ-GFP transfected cells, which could reflect ERβ cleaved from GFP. (E) H150 anti-ERβ detected a single band of inappropriate size (∼55 kDa) in all transfected cells. (F) 9.88 anti-ERβ detected a single band of inappropriate size (∼102 kDa) in all transfected cells. (G) Similar to H150, 06-629 anti-ERβ detected a band close to 52 kDa in all transfected cells, as well as band of ∼38 kDa in all transfected cells. (H) Y19 anti-ERβ detected faint bands in GFP and ERα-GFP transfected cells.

HT22 cells. Confocal imaging of anti-GFP staining showed diffuse, cytoplasmic labeling in GFP-only transfected cells (Fig. 3A1) and nuclear labeling for both ERα-GFP and ERβ-GFP transfected cells. PA1-310B showed no immunoreactivity in ERα-GFP transfected cells (Fig. 3A2) and nuclear labeling in ERβ-GFP transfected cells (Fig. 3A3). Results were identical for ck5912 (Fig. 3B). Thus, PA1-310B and ck5912 both appeared promising.

We next investigated immunolabeling in C57/B6 wildtype (WT) and βERKO mouse ovary. In contrast to Krege et al. (1998), we found that PA1-310B showed labeling in granulosa cells from both WT (Fig. 4A) and βERKO (Fig. 4B) ovaries, although staining was less intense in βERKOs. This surprising result prompted us to further investigate labeling in βERKO tissue.

We made whole cell extracts from WT and βERKO (KO) ovary, hypothalamus, and hippocampus and probed western blots with a similar panel of antisera as we used in previous specificity tests. While labeling was often faint in ovary samples, importantly, there was no apparent difference in either the molecular weight or intensity of any bands between WT and KO tissues with any antisera. PA1-310B, ck5912 and 9.88 detected a single band close to 52 kDa, while D7N detected a band slightly below and H150 detected a band slightly above 52 kDa in WT and KO tissue (Fig. 5A–E). 06-629 labeled multiple bands between 52 and 76 kDa in WT and KO tissues (not shown). Additionally, 1531 detected strong bands of ∼55 kDa in βERKO and WT ovary and, along with Y19 and L20, detected multiple faint bands in both
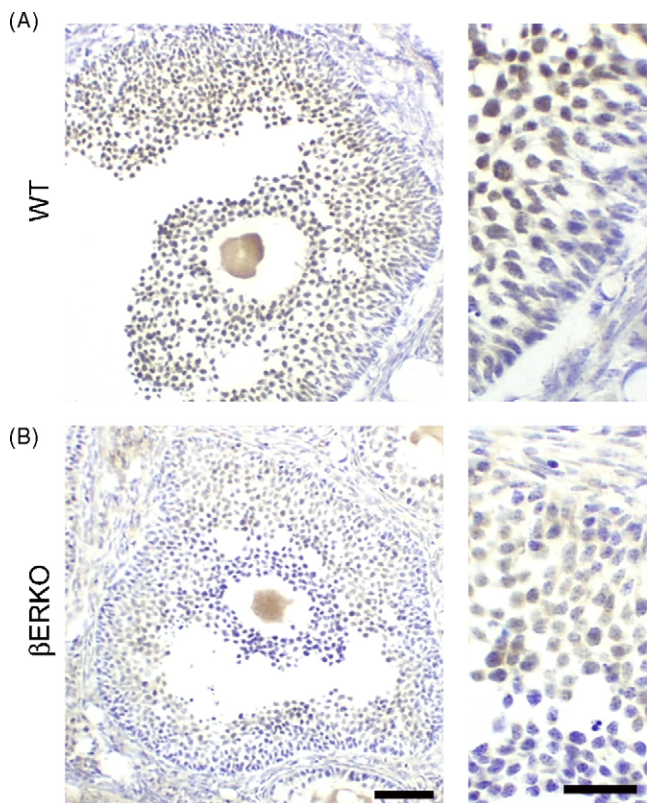
Fig. 3. Detection of ERβ in transfected HT22 cells by immunocytochemistry. (A) Representative images of PA1-310B immunostaining in HT22 cells transfected with (A_1) GFP, (A_2) ERα-GFP, or (A_3) ERβ-GFP. PA1-310B anti-ERβ detected nuclear labeling only in ERβ-GFP transfected cells, as appropriate. (B) Representative images of ck5912 immunostaining in HT22 cells transfected with (B_1) GFP, (B_2) ERα-GFP, or (B_3) ERβ-GFP. Ck5912 anti-ERβ detected nuclear labeling only in ERβ-GFP transfected cells, as appropriate. Scale bar is 10 μm and applies to all frames.

WT and βERKO hypothalamus and hippocampus (not shown). We further tested ck5912 by preadsorption with the immunogenic peptide. Ck5912 detected a single band at ∼50 kDa for rERβ and a band at ∼52 kDa in extracts from rat, WT, and βERKO mouse hypothalamus (Fig. 6A). In each case, labeling was completely eliminated by preadsorption of the antiserum with the immunogenic peptide (Fig. 6B). A similar experiment was performed for PA1-310B on rat tissue and preadsorption also eliminated staining (not shown).

Labeling in βERKO tissues was a serious concern. To exclude the possibility that residual ERβ was expressed in βERKO tissues, we performed rtPCR for ERβ mRNA on WT and βERKO ovary, hypothalamus, and hippocampus (Fig. 7A). DNA sequencing was then performed on WT and βERKO hypothalamus and hippocampus. The expected full-length product of 1291 bp was found in WT ovary, hypothalamus, and hippocampus and surprisingly, also

in βERKO hippocampus (Fig. 7A). βERKO ovary, hypothalamus, and hippocampus contained lower base pair products. Sequencing showed that while the WT sequences contained no stop codons and therefore were likely to be translatable, βERKO sequences contained stop codons. For hypothalamus, partial sequences of ∼940 bp were obtained for both WT and βERKO samples. While the WT hypothalamic sequence was translatable, the βERKO hypothalamic sequence contained 2 stop codons in exon 4 (Fig. 7B). For hippocampus, WT and the full-length βERKO product, which corresponded to the product from WT hippocampus (Fig. 7A), were sent for sequencing. A sequence of 708 bp was obtained for WT and 1,077 bp for βERKO hippocampus. Similar to hypothalamus, the WT hippocampal transcript contained no stop codons whereas the βERKO hippocampal transcript is not likely to be translatable due to a stop codon in exon 5 (Fig. 7B). Thus, rtPCR and sequencing analysis indicated that ERβ protein is not expressed in βERKO
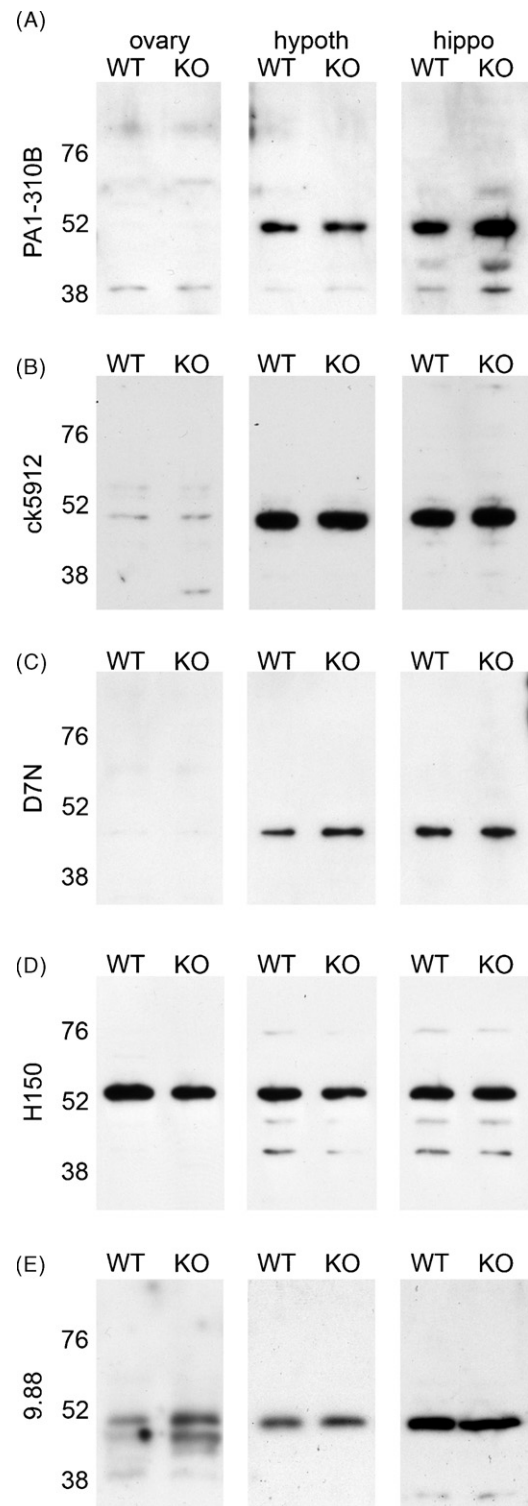
Fig. 4. ERβ immunoreactivity in wildtype (WT) and ERβ knockout mouse (βERKO) ovaries. Representative photomicrographs from (A) WT and (B) βERKO ovaries labeled with PA1-310B anti-ERβ. ERβ immunoreactivity is detected in granulosa cells of both WT and βERKO mice, although less intense in βERKO. Scale bars are 50 μm and 25 μm, left and right panels, respectively.

tissues. Interestingly, both hippocampal transcripts contained the 54 bp insert corresponding to ERβ2 (Fig. 7B).

To corroborate results with βERKO mice, we also made whole cell extracts from wildtype (WT) and ERβ null (ERβ$_{ST}$$^{L-/L-}$, Antal et al., 2008) mouse tissues and probed western blots with a panel of ERβ antisera. Similar to results with βERKO tissue, all of the ERβ antisera we tested recognized the same bands in tissue from WT and ERβ$_{ST}$$^{L-/L-}$ mice. PA1-310B detected prominent bands at ~52 and ~40 kDa in ovary, hypothalamic, and hippocampal extracts from both WT and ERβ$_{ST}$$^{L-/L-}$ (Fig. 8A). Ck5912 detected several bands in ovary, and a single band at ~52 kDa in WT and ERβ$_{ST}$$^{L-/L-}$ hypothalamic and hippocampal extracts (Fig. 8B). D7N detected a faint band of ~60 kDa in WT and ERβ$_{ST}$$^{L-/L-}$ ovary and a ~45 kDa band in hypothalamus and hippocampus (Fig. 8C). Similar to results from cell culture, H150 recognized a band of ~55 kDa in all extracts from WT and ERβ$_{ST}$$^{L-/L-}$ tissue (Fig. 8D). 9.88 detected multiple bands in ovary and a single band of ~55 kDa in WT and ERβ$_{ST}$$^{L-/L-}$ hypothalamic and hippocampal extracts (Fig. 8E). 06-629, 1531, Y19, and L20 detected multiple bands in WT and ERβ$_{ST}$$^{L-/L-}$ tissues (not shown). Thus, while all ERβ antisera detected a band or bands at molecular weights appropriate for ERβ, the observations of apparently identical labeling in WT and both βERKO and ERβ null tissues are impossible to overlook and raise serious concerns about the specificity of all 9 antisera we tested.

4. Discussion

We initially set out to characterize a new affinity-purified anti-ERβ antiserum produced in chicken, ck5912. We included 8 commercially available ERβ antisera as controls: Affinity Bioreagents PA1-310B, Invitrogen D7N, Upstate 06-629, Santa Cruz

Fig. 5. Anti-ERβ antisera recognize nearly identical bands on western blots from wildtype (WT) and ERβ knockout (βERKO, KO) mouse tissues. Representative western blots of ovary, hypothalamus (hypoth), and hippocampus (hippo) from WT and βERKO (KO) probed with (A) PA1-310B anti-ERβ, (B) ck5912 anti-ERβ, (C) D7N anti-ERβ, (D) H150 anti-ERβ, or (E) 9.88 anti-ERβ.
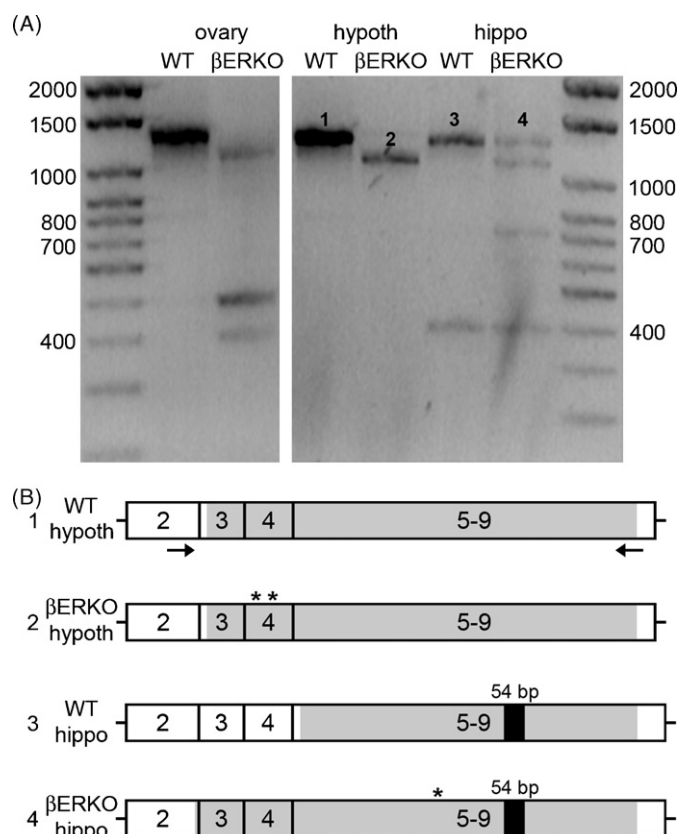
H150, L20, Y19, and 1531, and Abcam 9.88. We first tested recognition of recombinant ERα and ERβ, then detection of ERα and ERβ in transfected cell lines, followed by the most important test, labeling in tissues from WT versus βERKO and ERβ$_{ST}$$^{L-/L-}$ mice. During this process, we found that ck5912, along with the 8 control antisera,
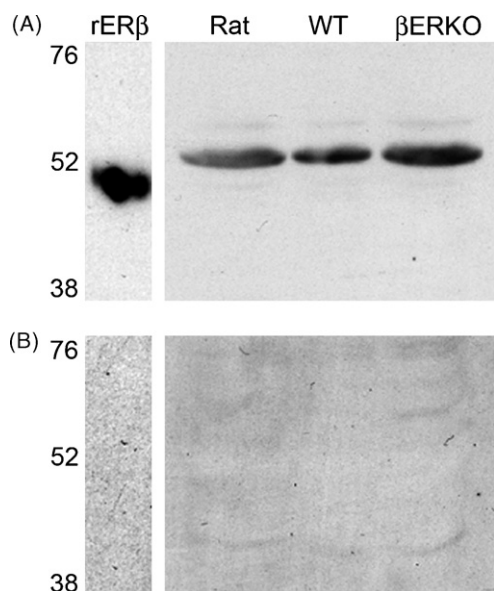
Fig. 6. ERβ immunoreactivity is eliminated by preadsorption of the antiserum with the antigenic peptide. (A) Representative western blot of rERβ, rat, wildtype and estrogen receptor beta knockout (βERKO) hypothalamic tissue probed with ck5912 anti-ERβ. One prominent band was detected for rERβ, rat, WT and βERKO tissues. (B) Preadsorption with the antigenic peptide eliminated all labeling.

Fig. 7. rtPCR for ERβ mRNA in wildtype (WT) and ERβ knockout mouse (βERKO) ovary, hypothalamus (hypoth), and hippocampus (hippo). (A) Representative gel electrophoresis of the rtPCR products. WT ovary, hypothalamus, and hippocampus and βERKO hippocampus contained the expected full-length product of 1291 bp. Additionally, βERKO ovary, hypoth, and hippo contained lower base pair products. Numbers indicate the transcripts sent for sequence analysis. (B) Representation of the rtPCR products from WT and βERKO mRNA that were sequenced showing exons 2–9. The black arrows indicate the primers used, the shaded regions indicate the portion for which sequence data were obtained, and asterisks indicate stop codons. Sequences obtained from WT hypoth and hippo PCR products did not contain stop codons and are therefore likely to be translated. However, both βERKO hypoth and hippo products contained stop codons. Interestingly both hippocampal samples contained the 54 bp insert corresponding to ERβ2.

recognized apparently identical protein bands by western blot in WT, βERKO, and ERβ$_{ST}^{L-/L-}$ ovary, hypothalamus, and hippocampus.

To date, 5 lines of ERβ-deficient mice have been produced. The first βERKO mouse was originally generated by Krege et al. (1998); this same line was duplicated at the Karolinska Institute and used in studies of bone (Windahl et al., 1999), brain (Wang et al., 2001, 2003), and prostate (Imamov et al., 2004). Three other independent lines were also made, by Chambon and colleagues (Dupont et al., 2000), by Shughrue et al. (2002) at Wyeth, and the ERβ$_{ST}^{L-/L-}$ line also by Chambon's group (Antal et al., 2008). Given an initial focus on the role of ERβ in reproductive function, the original characterizations of ERβ-deficient mice rarely investigated ERβ expression in brain. One exception was the mouse produced at Wyeth. Using the Z8P antiserum that is no longer available, Shughrue et al. (2002) showed a lack of ERβ immunoreactivity in the hypothalamic paraventricular nucleus, a region that expresses ERβ intensely in WT mice. Also, using this same mouse, a lack of ERβ in brain capillary endothelial cells was reported based on western blots probed with the D7N antiserum (Razandi et al., 2004). Unfortunately, the Wyeth mouse is currently unavailable for further experiments. Still, subsequent studies utilizing the Krege et al. (1998) βERKO mouse have shown several neural differences from WT that suggest ERβ expression in brain is disrupted, including alterations in neuronal migration, hypocellularity in regions known to express ERβ mRNA, as well as deficits in social recognition and spatial learning (Wang et al., 2001, 2003; Rissman et al., 2002; Choleris et al., 2003). These findings, along with its commercial availability, made the βERKO mouse a good choice for characterization of the ck5912 antiserum. We also used tissues from the ERβ null mouse, ERβ$_{ST}^{L-/L-}$, to confirm results obtained with βERKO mice. As with βERKOs, the original analysis of ERβ expression in ERβ$_{ST}^{L-/L-}$ mice focused on reproductive tissues. rtPCR on ERβ$_{ST}^{L-/L-}$ uterine and ovarian tissue showed no ERβ transcripts containing sequences beyond exon 3, and a frame shift-induced stop codon at nucleotide 599 predicts a severely truncated protein (Antal et al., 2008). Western blots of testis and prostate tissue probed using an antiserum raised to aa 465–485 of ERβ showed multiple bands in both WT and ERβ$_{ST}^{L-/L-}$,

but notably, ERβ$_{ST}^{L-/L-}$ tissues lacked a band at 61 kDa that was present in WT (Antal et al., 2008).

The antisera we tested recognized protein bands ranging from 45 to 60 kDa, within the range of known ERβ isoforms (LaVoie et al., 2002; Lewandowski et al., 2002). These antisera were generated against a diverse set of epitopes and were produced in various host species including, rabbit, mouse, chicken, and goat (Table 1). It is therefore highly unlikely that the labeling seen in βERKO and ERβ$_{ST}^{L-/L-}$ tissues is an artifact of secondary reagent labeling of IgG bands. Most of the antisera we tested recognized rERβ and not rERα, giving an initial impression of specificity. However two of them, H150 and 06-629, also recognized rERα. Both H-150 and 06-629 were raised against an N-terminal sequence in the A/B domain of ERβ, which shares low sequence homology with ERα (Kuiper et al., 1996; Tremblay et al., 1997). Therefore, the source of their cross-reactivity with ERα is unclear. In the case of H150, it is unlikely that ERα accounts for the labeling seen in tissue, since the immunolabeled band was consistently ∼55 kDa, too low for ERα (68 kDa).

In contrast to other antisera, PA1-310B and ck5912 initially looked promising based on specificity for rERβ and ERβ transfected into HT22 or HEK 293 cells. However, in subsequent tests, these and other antisera showed identical immunoreactivity in WT compared with βERKO and ERβ$_{ST}^{L-/L-}$ mice. The original charac-
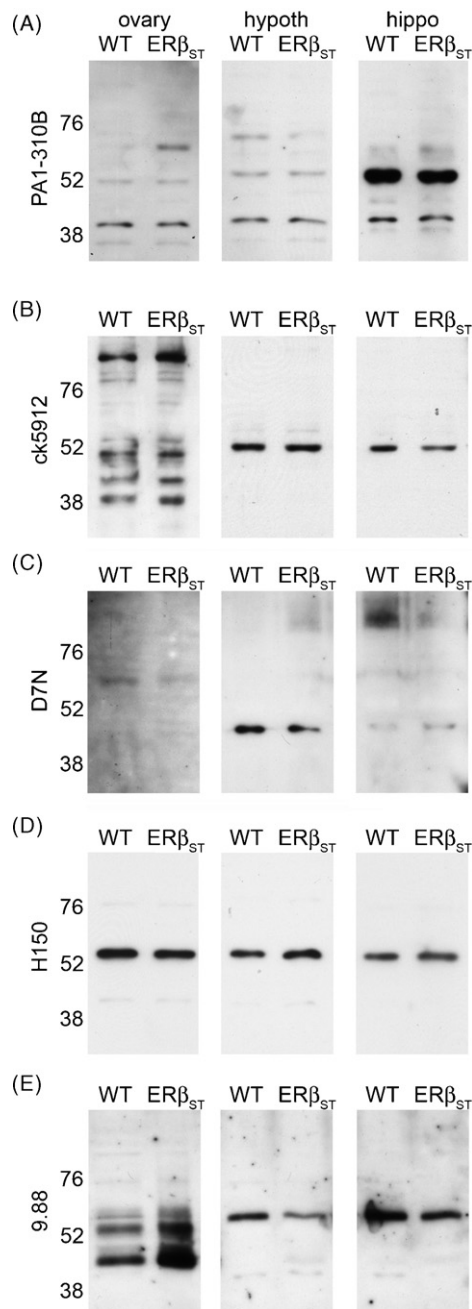
Fig. 8. Anti-ERβ antisera recognize nearly identical bands on western blots from wildtype (WT) and ERβ null (ERβ$_{ST}^{L-/L-}$, ERβ$_{ST}$) mouse tissues. Representative western blots of ovary, hypothalamus (hypoth), and hippocampus (hippo) from WT and ERβ$_{ST}$ mice probed with (A) PA1-310B anti-ERβ, (B) ck5912 anti-ERβ, (C) D7N anti-ERβ, (D) H150 anti-ERβ, or (E) 9.88 anti-ERβ.

terization of the βERKO mouse reported a lack of nuclear labeling in ovarian granulosa cells using PA1-310B anti-ERβ (Krege et al., 1998). Yet, in our experiments we saw faint immunolabeling using this same antiserum. It is possible that variation between lots of antiserum and/or in the experimental protocols used accounts for this discrepancy (Lorincz and Nusser, 2008).

The βERKO mouse was generated by disrupting exon 3 of ERβ and the ERβ$_{ST}^{L-/L-}$ null mouse was generated by excising exon 3. Because a splice variant of ERβ that lacks exon 3 (ERβΔ3) has been characterized in rat and human (Petersen et al., 1998; Poola et al., 2002), it was important to exclude the possibility that an ERβΔ3 variant was expressed in βERKO tissues, which might account for the immunolabeling we observed. In previous work, rtPCR was per-

formed on βERKO ovarian and prostate tissue and this revealed transcript variants encoding truncated proteins (Krege et al., 1998). We performed a similar experiment as Krege et al. (1998) on WT and βERKO ovary, and because neither Krege et al. (1998) nor Antal et al. (2008) investigated ERβ expression in brain, we also included hypothalamus and hippocampus. Sequence analysis showed that neither the βERKO hypothalamic nor hippocampal transcripts were likely to account for protein bands of apparently identical molecular weight and intensity in WT and βERKO extracts. Both hypothalamic and hippocampal βERKO transcripts contained stop codons that would lead to severely truncated proteins. Thus, while it is conceivable that a truncated ERβ variant could be expressed in βERKOs, one would expect to see a clear difference in molecular weight(s) and we observed no differences. It also seems very unlikely that all of the antisera we tested recognize specifically an ERβΔ3 isoform. The more likely explanation for our results is that an as yet uncharacterized protein similar to ERβ cross-reacts with many ERβ antisera.

Our results are in stark contrast to those obtained with the Z8P antiserum, which as noted above, did not label in tissues from ERβ deficient mice (Shughrue et al., 2002). Thus, based on this test and the good correspondence between Z8P immunoreactivity and ERβ mRNA, it is likely that brain areas labeled with Z8P do express ERβ protein. Shughrue and Merchenthaler (2001) used Z8P to generate a comprehensive atlas of nuclear ERβ immunoreactivity in the brain and reported labeling in many brain areas including (but not limited to) strong labeling in the preoptic area and hypothalamus, bed nucleus of the stria terminalis, and amygdala, moderate labeling in the neocortex and hippocampus, as well as in areas of the mid- and hindbrain. In addition to nuclear labeling, Z8P also has been used to show extranuclear ERβ immunoreactivity both *in vitro* and *in vivo* (Kalita et al., 2005; Milner et al., 2005; Jelks et al., 2007). Thus, it remains a strong possibility that ERβ is responsible for at least some of the rapid effects of estradiol in the brain.

We are not the first to report a lack of specificity in commercially available antisera. For example, Grimsey et al. (2008) tested several commercially available anti-cannabinoid CB$_1$ receptor antisera and found that they failed to specifically immunolabel CB$_1$ receptor transfected into HEK 293 cells. Similar to our findings, several commercially available antisera for muscarinic receptors have been shown to label identically in tissue from wildtype and muscarinic receptor knockout mice by western blot and immunohistochemistry (Pradidarcheep et al., 2008). Additionally, multiple galanin receptor antisera were reported to label in galanin receptor knockout mice (Lu and Bartfai, 2009). These reports, along with our own study on ERβ antisera, highlight the need for careful characterization of antisera used for protein localization or functional studies.

In conclusion, our results indicate that, while many ERβ antisera do recognize rERβ and ERβ expressed in cultured cells, they also recognize some protein(s) other than known ERβ variants *in vivo* that is/are present in βERKO and ERβ$_{ST}^{L-/L-}$ mouse brain. Thus, the results of experiments utilizing these antisera should be interpreted with appropriate caution.

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