Distribution of Two Isozymes of 5α-Reductase in the Brains of Adult Male and Female Green Anole Lizards

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Key Words
Amygdala • Androgen metabolism • Preoptic area • Testosterone • Ventromedial hypothalamus

Abstract
The 5α-reductase (5αR) enzyme converts testosterone to 5α-dihydrotestosterone. This local metabolism within the brain is important for the full expression of male sexual behavior in many species, including green anole lizards. Two isozymes of 5αR exist and little is known about their specific distributions. We conducted in situ hybridization for both isozymes in intact male and female green anole brains during the breeding (BS) and non-breeding (NBS) seasons. 5αR1 mRNA was only detected in the brainstem, while 5αR2 was expressed in specific areas throughout the brain. As our primary interest was evaluating the potential role of 5αR in forebrain regulation of reproductive behavior, we quantified 5αR2 expression in the preoptic area, amygdala (AMY), and ventromedial hypothalamus (VMH). More 5αR2 cells were detected during the NBS than BS in the AMY, and the density of these cells was greater in females than males. In the VMH, the right side contained more 5αR2 cells than the left, an effect driven by a lateralized increase in the NBS. These data expand understanding of the distribution and potential roles of both isozymes in the adult brain, and differences in expression patterns between mammals and birds suggest that they may have been co-opted for different functions later in evolution.

Introduction
Steroid hormones are important for the expression of sexual behaviors in many vertebrate species [Ball and Balthazart, 2002; Fabre-Nys and Martin, 1991; Fusani, 2008; Hull and Dominguez, 2007; Steel, 1981; Tennent et al., 1980]. These hormones act at brain regions that are critical for the expression of sexual behaviors, including the preoptic area (POA) and amygdala (AMY), which control male actions such as courtship and copulatory behaviors [Balthazart and Surlemont, 1990; Crews and Moore, 2005; Kostarczyk, 1986; Thompson et al., 1998; Tobet et al., 1986], and the ventromedial hypothalamus (VMH), which controls female behaviors such as receptive and receptive behaviors [Emery and Moss, 1984; La Vaque and Rodgers, 1975; Wade and Crews, 1992]. Neural metabolism of gonadal hormones is often critical. For example, local conversion of testosterone (T) into estradiol (E2) and 5α-dihydrotestosterone (DHT) facilitates
iors in many organisms, including Japanese quail, hamsters, rats, and guinea pigs [Balthazart, 1991; Hull and Domínguez, 2007; Romeo et al., 2001]. Aromatase is the enzyme that converts T to E2, and 5α-reductase (5αR) converts T to DHT. While much is known about aromatase [reviewed in Lephart, 1996], much less information is currently available for 5αR.

Two isozymes of 5αR exist: 5αR1 and 5αR2 [Lephart et al., 2001]. In mammals, neural 5αR1 is generally more abundant than 5αR2 [Celotti et al., 1997]. Expression is greater overall for both isozymes in the brainstem than in the forebrain, however, 5αR2 is found in hypothalamic and hippocampal neurons in adult rodents [Paletti and Martini, 1999]. 5αR1 has a lower affinity for T and is present in both neurons and glial cells, whereas 5αR2 has a higher affinity for T and is found primarily in neurons [Negri-Cesi et al., 2008]. Thus, these 2 isozymes may serve somewhat different functions.

In general, whole-brain 5αR activity does not differ between males and females, and is not typically modulated by steroid hormones. However, evidence in adult rats suggests that 5αR2 mRNA, but not 5αR1, can be upregulated by T [Negri-Cesi et al., 1996; Torres and Ortega, 2003, 2006]. Specific distributions and relative patterns of expression of 5αR1 and 5αR2 in areas likely to control reproductive behaviors are unknown. Additionally, expression of these isozymes has not yet been examined in reptilian species.

Green anole lizards are seasonally breeding animals native to the southeastern United States, with the breeding season (BS) lasting from approximately April to August. During the non-breeding season (NBS) gonads regress and circulating steroid hormone levels decrease [Lovern et al., 2001]. Thus, these animals offer an excellent natural experiment to examine how circulating T levels across the year might influence 5αR1 and 5αR2 expression.

Similar to other species, DHT is important for the full expression of male reproductive behaviors [Mason and Adkins, 1976; Rosen and Wade, 2000]. 5αR activity in anoles is also similar to what has been observed in other species. Activity is higher in the brainstem than the forebrain, and whole brain activity does not differ between the sexes or the seasons in gonadally intact animals [Rosen and Wade, 2001; Wade, 1997]. However, T treatment increases whole-brain 5αR activity in gonadectomized males [Cohen and Wade, 2010].

Although it is known that 5αR activity is concentrated in the brainstem of many species, including anoles [Wade, 1997], detailed expression patterns have not been reported. Thus, this experiment was designed to determine the distribution of both isozymes of 5αR in the green anole brain, and to investigate local changes in expression due to sex and season. This study also begins to address the hypothesis that T can influence the distribution of the 2 isozymes by examining the brain under naturally occurring differences in T levels (male vs. female, BS vs. NBS).

Methods

Animals and Tissue Processing

Male and female green anole lizards were purchased from Charles Sullivan Co. (Nashville, Tenn., USA) during the BS (May) and NBS (November). These animals were wild-caught and sent to our animal facilities within a few days of capture. Animals were housed individually in 10-gallon aquaria. Peat moss was provided as substrate, and cages contained rocks, sticks, and water dishes. Aquaria were misted daily, and crickets dusted with calcium phosphate powder were provided 3 times (BS) or 2 times (NBS) per week. During the BS, animals were kept on a 14:10 light/dark cycle and ambient temperatures ranged from 28°C during the day to 19°C at night. During the NBS, animals were kept at a 10:14 light/dark cycle and ambient temperatures ranged from 24°C during the day to 15°C at night. Heat lamps and full-spectrum lights were provided above the cages, which allowed the animals to bask at temperatures 10°C above ambient temperatures. Relative humidity was maintained at approximately 70% throughout both seasons.

At least 2 weeks after arrival in the lab, animals were rapidly decapitated. Breeding state was confirmed at this time, with animals in the NBS having fully regressed gonads. Males in the BS had large, vascularized testes, and females had at least 1 yolking follicle. Brains were collected, immediately frozen in methyl butane on dry ice, and stored at −80°C until processing. Brains were sectioned coronally at 20 μm into 4 alternate series (3 were used for the present study, see below) and thaw-mounted onto SuperFrost plus slides (Fisher Scientific, Hampton, N.H., USA). Slides were stored with dessicant at −80°C until further processing.

All procedures adhered to NIH guidelines and were approved by the Michigan State University Institutional Animal Care and Use Committee.

Cloning of 5αR1 and 5αR2

Testes were taken from 2 breeding males and homogenized in Trizol (Invitrogen Corp., Carlsbad, Calif., USA). RNA was separated from the homogenate using chloroform, and then isolated using RNeasy minicolumns (Qiagen Sciences, Valencia, Calif., USA) and concentrated using ethanol precipitation. It was reconstituted in DEPC-treated water and stored at −80°C. RNA was converted into cDNA with the SuperScript III reverse transcriptase kit (Invitrogen) per the manufacturer’s instructions, and stored at −20°C until use.

Anole-specific 5αR1 and 5αR2 cDNA sequences were found using the Ensembl Genome Browser (ENSACAG00000000672 and ENSACAG00000000551, respectively). The top 25 results from a BLASTx (NCBI) search of the green anole 5αR1 represented diverse vertebrates with 53–61% sequence identity. Parallel
results were obtained for 5αR2, with somewhat higher identity (57–79%). Primers were designed based on the anole sequences for both genes using the Oligo Analysis Tool program (Eurofins MWG Operon, Huntsville, Ala., USA; table 1) and purchased from Invitrogen. PCR reactions included 1 U Platinum Taq High Fidelity DNA polymerase (Invitrogen), 0.2 mM dNTP mixture, 0.2 μM primer mix, 2 mM MgSO₄, and template cDNA from the testes. The PCR reaction went through 40 cycles at 94 °C for 15 s, 55 °C for 30 s, and 68 °C for 1 min. 5αR2 was amplified once, purified using the QIAquick PCR purification kit (Qiagen) and concentrated using ethanol precipitation. 5αR1 was amplified twice, using the same primers and the product from the first reaction as the template for the second. The pGEM-T Easy Vector System I (Promega Corp., Madison, Wisc., USA) was used to ligate the A-tailed PCR products to vectors as per manufacturer’s instructions.

We transformed One Shot TOP10 Chemically Competent E. coli cells (Invitrogen) with the ligated vectors and grew them on LB agar plates containing 100 μg/ml of ampicillin and 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-Gal). White colonies were selected and grown overnight in LB broth containing 100 μg/ml of ampicillin. Vector DNA was isolated using Wizard Plus Miniprep kits (Promega), and the sequences of the inserts were confirmed in both directions on a Perkin Elmer/Applied Biosystems 3100 capillary sequencer. After sequence confirmation, vector DNA was isolated using Wizard Plus Maxiprep kits (Promega) and stored at −20°C.

Table 1. Primers used to clone 5αR1 and 5αR2 from the green anole. Melting temperatures (Tₘ) are indicated

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5’ to 3’)</th>
<th>Tₘ (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5αR1 forward</td>
<td>TGATGGTGGCGCTGAGCAA</td>
<td>67.73</td>
</tr>
<tr>
<td>5αR1 reverse</td>
<td>TTCCCTTCTTCGATAGT</td>
<td>56.67</td>
</tr>
<tr>
<td>5αR2 forward</td>
<td>CTTGTTCCGAGGAGTT</td>
<td>57.86</td>
</tr>
<tr>
<td>5αR2 reverse</td>
<td>GGATACGTGATATGT</td>
<td>55.38</td>
</tr>
</tbody>
</table>

In situ Hybridization

Briefly, sense (SP6) and antisense (T7) probes were transcribed for both genes using the Digoxigenin RNA Labeling Kit per manufacturer’s instructions (Roche Diagnostics; Indianapolis, Ind., USA), which labels RNA with digoxigenin-UTPs. Probes were cleaned using a G50 sephadex bead column and stored at −80°C until use. For each gene, 1 set of slides from each animal was used for the antisense reaction. As a control, another set of slides from 1 animal from each group was used for the sense reaction. Slides were thawed and then fixed for 10 min in 4% paraformaldehyde in 0.1 M PBS (pH 7.4). They were treated with 0.25% acetic anhydride in triethanolamine-HCl with 0.9% NaCl buffer (pH 8.0). Slides were then incubated overnight at 45°C with 200 ng/ml 5αR1 or 100 ng/ml 5αR2 probe in hybridization buffer, which consisted of 50% formamide, 4× SSC, 1× Denhardt’s solution, 200 μg/ml fish sperm DNA, 10% dextran sulfate, 20 mM dithiothreitol, 250 μg/ml tRNA, 2 mM EDTA, and 0.1% Tween-20. The next day, slides were rinsed in 2× SSC and 0.2× SSC at 60°C.

They were then treated with 0.9% H₂O₂ in maleic acid buffer (pH 7.5) with 0.1% Tween-20 (MABT) for 30 min. They were incubated in a blocking solution of 5% normal sheep serum (Jackson Immuno Research, West Grove, Pa., USA) in MABT for 30 min, and were treated with 0.5 μl/ml Anti-Digoxigenin-AP Fab fragments (Roche) in MABT. After 2 h, the color reaction was conducted by incubating the slides with 4.5 μl/ml NBT and 3.5 μl/ml BCIP (Roche) in 0.1 M Tris-HCl and 0.1 M NaCl (pH 9.5). Reaction time was titrated so that the slides incubated with the antisense probe showed distinct reaction product within the cytoplasm of individual cells with an absence of labeling on the sense-treated slides (about 9 min for 5αR1 and 5 min for 5αR2; fig. 1), the color reaction was stopped with 1 M Tris and 0.5 M EDTA (pH 8.0).

5αR1 and 5αR2 Mapping

We mapped the expression of both 5αR1 and 5αR2 in the brain. Regions in the anole forebrain were identified using a green anole atlas [Greenberg, 1982]. Brainstem regions were identified using ten Donkelaar and Nieuwenhuys [1979] and Barbas-Henry and Lohman [1984]. Three different labeling intensities were determined: light, medium and dark (fig. 1). The light labeling was characterized by the blue reaction product that was confined to the cytoplasm, but was somewhat punctuated and did not fill the entire cellular compartment. Dark labeling was characterized by labeling that was intense and specifically filled the entire cytoplasm of individual cells. Medium labeling was intermediate: the labeling was clearly cytoplasmic, filled most of that portion of the cell and was darker than the light labeling.

5αR2 Quantification

Because 5αR1 was not expressed in the forebrain regions mediating sexual behavior (POA, AMY, and VMH), only 5αR2 was quantified. These 3 regions were chosen because previous work has documented that exhibit sexual and seasonal dimorphisms
sopharyngeal portion of the nucleus ambiguus and the ventral motor nucleus of the facial nerve (Amb IX/VII) and reticular nucleus (R1). In contrast, dark 5αR2 staining was detected in specific regions throughout much of the brain, including those regions important in controlling sexual behaviors (POA, AMY, and VMH; fig. 3). 5αR2 was lightly expressed in the forebrain, and exhibited much higher intensity in the brainstem. The darkest labeling of 5αR2 expression was observed in nIII/IV, Amb IX/VII, and nVI.

**5αR2 in the POA**

We detected no effects of, or interactions between, sex and season on the number of 5αR2-expressing cells (all \( F < 3.12, \ p > 0.090; \) table 2). No effects were detected on the density (cell number per unit volume) for any of these variables (all \( F < 1.59, \ p > 0.219 \)).

Similar to previous work, the volume of the POA was larger in males compared to females (\( F = 12.51, \ p = 0.002; \) table 3).

**5αR2 in the AMY**

No effects were detected on the number of 5αR2 expressing cells (all \( F < 3.70, \ p > 0.07, \) fig. 4), although on average more 5αR2 expressing cells were present in the NBS. A sex difference was detected in the density of 5αR2 cells, such that more were present per unit volume in females compared to males (\( F = 4.61, \ p = 0.046; \) fig. 4). No other main effects or interactions were detected for this measure (all \( F < 0.04, \ p > 0.847 \)).

We also found a sex difference in the volume of the AMY; the region was larger in males than in females (\( F = 6.47, \ p = 0.02; \) table 3).

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**Table 2. 5αR2 expression in the POA**

<table>
<thead>
<tr>
<th></th>
<th>Number</th>
<th>Density, cells/mm(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>BS</td>
<td>6,018 (1,050)</td>
</tr>
<tr>
<td></td>
<td>NBS</td>
<td>7,670 (611)</td>
</tr>
<tr>
<td>Male</td>
<td>BS</td>
<td>8,334 (739)</td>
</tr>
<tr>
<td></td>
<td>NBS</td>
<td>8,055 (412)</td>
</tr>
</tbody>
</table>

Data show estimated total number and density of positive cells. Values are means (SE). No effects of sex, season or interaction were detected.
Fig. 2. Camera lucida drawings depicting patterns of 5αR1 and 5αR2 expression throughout the anole brain (the distribution was symmetrical; only half the brain is depicted). Open circles = Light labeling; grey circles = medium labeling; dark circles = dark labeling. Acc = Nucleus accumbens; AC = anterior commissure; ADVR = anterior dorsal ventricular ridge; Amb X = vagal portion of nucleus ambiguus; Amb IX/VIIvm = glossopharyngeal portion of the nucleus ambiguus and the ventral motor nucleus of the facial nerve; AMY = amygdala; BNac = bed nucleus of the anterior commissure; BNhc = bed nucleus of the hippocampal commissure; BNst = bed nucleus of the stria
tum; Cx = central canal; CxD = dorsal cortex; CxL = lateral cortex; CxM = medial cortex; DH = dorsal horn of the spinal cord; FLM = medial longitudinal fasciculus; LFB = lateral forebrain bundle; MMN = mammillary nucleus; MP = medial pretectal nucleus; nIII/IV = oculomotor and trochlear nuclei; nV = trigeminal nucleus; nVI = abducens nucleus; nXI/XII = accessory and hypoglossal nuclei; OPT = optic tract; OT = optic tectum; PHN = posterior hypothalamic nucleus; POA = preoptic nucleus; RAI = inferior raphe nucleus; RI = reticular nucleus; RUB = red nucleus; RS = superior reticular nucleus; Sep = septum; Str = striatum; TC = tectal commissure; Thal = thalamus; Tor = torus semicircularis; VH = ventral horn of the spinal cord; VMA = ventromedial area; VMH = ventromedial hypothalamus.
The estimated total number of cells that expressed 5αR2 in this brain region was lateralized (F = 5.60, p = 0.030; fig. 5), such that the right side expressed more 5αR2 than the left. There was also an interaction between side and season (F = 5.89, p = 0.027), which was driven by an increase in 5αR2 expression in the right lateral VMH during the NBS (F = 9.24, p = 0.007). The expression in the left VMH did not change with season (F = 0.65, p = 0.431).

Parallel to what we found for the number of cells, the density of 5αR2 expressing cells was lateralized (F = 5.06, p = 0.038; fig. 5), again with the right VMH containing more than the left. No other effects were detected on all density measures (all F < 2.32, 3.15, p > 0.146).

Similar to previous work [unpublished observations], we found that males had a larger lateral VMH than females (F = 15.68, p = 0.001; table 3). We also found a seasonal difference in volume, such that the lateral VMH is larger during the NBS than BS (F = 11.01, p = 0.004).

**Discussion**

We report here for the first time the distribution of 2 isozymes of 5αR in the brain of a reptile. The patterns of distribution for 5αR1 and 5αR2 differed from one another. 5αR1 was detected only in the brainstem, while 5αR2 was found in particular regions throughout the brain, although it was concentrated in the brainstem. This result is consistent with previous work showing higher 5αR activity in the brainstem than the rest of the brain in these lizards [Wade, 1997]. Thus, it appears that, in the anole, both 5αR1 and 5αR2 contribute to the high level of activity detected in the brainstem, while the majority of activity detected throughout the rest of the brain is due to 5αR2. Our results also parallel data suggesting that the mammalian brainstem expresses higher levels of both isozymes than the forebrain [Celotti et al., 1997].

**Fig. 3.** Representative photographs highlighting the distributions of 5αR1 (left column) and 5αR2 (right column). The regions are depicted from rostral to caudal: POA (a, b), AMY (c, d), VMH (e, f), nIII (g, h), and Amb IX/VIImv (i, j). 5αR1 is only present in the brainstem nuclei, while 5αR2 is present throughout the brain. Arrows indicate the boundary of the brain regions. Scale bar = 100 μm (a–h) or 50 μm (i, j). OT = Optic tract; 3V = 3rd ventricle; 4V = 4th ventricle.
The fact that both 5αR1 and 5αR2 are expressed in the brainstem of lizards as well as mammals, suggests a potentially conserved function for this enzyme in that portion of the brain.

Expression of these 2 isozymes has not been completely mapped in other species. In adult rats, 5αR1 mRNA is expressed at higher levels in whole brain homogenates, than 5αR2 mRNA [Negri-Cesi et al., 1996; Poletti et al., 1998]. Immunohistochemistry in rats for 5αR1 has detected the protein in the limbic system, as well as the cortex [Pelletier et al., 1994; Tsuruo et al., 1996]. 5αR2 was not investigated in the same way, although 5αR2 is expressed in the cortex in rats [Sanchez et al., 2008] and transiently expressed at higher levels than 5αR1 in homogenates of the entire brain during early development [Poletti et al., 1998]. In birds, 5αR activity has been detected throughout the brain, including the song control system, although the assay technique does not distinguish between the different isozymes [Balthazart, 1991].

It has been suggested that these enzymes play different roles in mammals: 5αR1 may serve to catabolize excess T, whereas 5αR2 may be important for sexual differentiation and male-specific behaviors [Melcangi et al., 1998]. Because it is not expressed as prevalently in the lizard brain, it is possible that 5αR1 was co-opted for clearing excess androgen from the brain later in evolution. In anoles, T itself is more important for the expression of male sexual behaviors, while the metabolites of T (estradiol and DHT) are more important for these behaviors in mammalian and avian species [Bakker et al., 2004; Mason and Adkins, 1976; Schlinger, 1997]. Thus, 5αR1 may not be as abundant in the anole brain, as compared to the mammalian brain, because T itself is the more active hormone.

**Fig. 4.** 5αR2 expression in the AMY in a female (a) and a male (b). A greater number of 5αR2 cells were detected during the NBS than BS (c). Females had a greater density of 5αR2-expressing cells than males (d). Dark grey bars = males; light grey bars = females. Scale bar = 50 μm.
In the present study, 5aR2 expression did not differ between the sexes or seasons in the POA or the VMH, which suggests that 5aR2 may not be important for the sexually dimorphic behaviors regulated by these areas. We did detect a lateralized effect of season on the number of 5aR2-positive cells increasing during the NBS. Parallel to the number of 5aR2 cells, the right side had a denser population of 5aR2 cells than the left. Dark grey bars = males; light grey bars = females.

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Table 3. Volume (mm$^3$) of 3 regions important for sexual behavior as defined by 5aR2 expression

<table>
<thead>
<tr>
<th></th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>POA</td>
<td>BS 0.021(0.001)</td>
<td>0.014(0.002)</td>
</tr>
<tr>
<td></td>
<td>NBS 0.021(0.002)</td>
<td>0.017(0.001)</td>
</tr>
<tr>
<td>AMY</td>
<td>BS 0.019(0.001)</td>
<td>0.015(0.001)</td>
</tr>
<tr>
<td></td>
<td>NBS 0.020(0.002)</td>
<td>0.017(0.001)</td>
</tr>
<tr>
<td>VMH</td>
<td>BS 0.023(0.002)</td>
<td>0.018(0.001)</td>
</tr>
<tr>
<td></td>
<td>NBS 0.028(0.001)</td>
<td>0.022(0.002)</td>
</tr>
</tbody>
</table>

Values are means (SE). All regions were larger in males than in females, regardless of season.

Similar to previous work [Beck et al., 2008, unpubl. obs. based on labeling of aromatase mRNA], we found that male anoles have a larger POA and lateral VMH than females. We detected a novel sex difference in AMY size (males larger than females) as defined by 5aR2 expression, which suggests that the borders defined by 5aR2 mRNA labeling compared to Nissl staining and aromatase mRNA are not exactly the same [Beck et al., 2008]. Because the 5aR2-expressing cells existed in greater density in females and without a sex difference in their overall number, the larger volume of the AMY in males is most likely due to cells that are more spread out. This change might reflect increased arborization, which could be evaluated in future studies.
We also detected a seasonal difference in the VMH, with the volume of the region larger during the NBS than the BS. Because the number of cells expressing 5αR2 is equivalent across the seasons, perhaps the cells are further spread apart during the NBS. Additionally, we detected a lateralized expression of 5αR2 in the VMH, although the function of this lateralization also remains unclear. We previously identified lateralized expression of aromatase in the AMY [unpubl. obs.]. Unlike eutherian mammals, reptiles and birds do not have a corpus callosum, and the absence of this structure limits the extent of communication between the 2 hemispheres of the brain [Mihrshahi, 2006]. This limited communication may allow for more lateralized functions of reptilian hypothalamic regions that should be further investigated.

Conclusions

To our knowledge, this is one of the first studies to examine expression of both isozymes in discrete brain regions in vertebrates and the first to examine the pattern in a reptile. The present data show that 5αR1 is mainly present in the brainstem, while 5αR2 is more widespread in the anole brain. This distribution pattern departs from what has been reported in mammals, where 5αR1 is the isozyme that is expressed at higher levels throughout the brain. It is possible that these 2 isozymes have different functions in the anole than in mammalian species. However, in both groups the brainstem expresses the highest levels of these isozymes, which suggests that there may be a similar function for 5αR in that portion of the brain across species. Expression of 5αR2 in the POA and VMH did not differ between the sexes and seasons, although we did find that 5αR2 expression was higher in the AMY of NBS animals and females. This suggests that 5αR2 may act to break down excess T in those groups that do not need high amounts of T in the AMY, or produce sufficient amounts of DHT during times when T levels are low.

Collectively, our results highlight the importance of examining these enzymes in multiple taxa. These data help expand current understanding of the roles for both isozymes in the adult brain, and differences in expression patterns with mammals suggest that these enzymes may have been co-opted for different functions later in evolution.

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References


