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QUANTITATIVE RT-PCR ANALYSES OF FIVE EVOLUTIONARY CONSERVED GENES IN ALLIGATOR BRAINS DURING DEVELOPMENT

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Abstract

Gene expression was investigated in the major brain subdivisions (telencephalon, diencephalon, midbrain and hindbrain) in a representative reptile, *Alligator mississipiensis*, during the later stages of embryonic development. The following genes were examined: voltage-gated sodium channel isoforms: NaV1.1 and NaV1.2; synaptic vesicle 2a (SV2a); synaptophysin; and calbindin 2. With the exception of synaptophysin, which was only expressed in the telencephalon, all genes were expressed in all brain regions sampled at the time periods examined. For NaV1.1, gene expression varied according to brain area sampled. When compared with NaV1.1, the pattern of NaV1.2 gene expression differed appreciably. The gene expression of SV2a was the most robust of any of the genes examined. Of the other genes examined, although differences were noted, no statistically significant changes were found either between brain part or time interval. Although limited, the present analysis is the first quantitative mRNA gene expression study in any reptile during development. Together with future experiments of a similar nature, the present gene expression results should determine which genes are expressed in major brain areas at which times during development in *Alligator*. When compared with other amniotes, these results will prove useful for determining how gene expression during development influences adult brain structure.

Keywords

Alligator • Calbindin 2 • qPCR • Synaptic vesicle protein 2 • Synaptophysin • Voltage-gated sodium channel

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1. Introduction

Despite having different alignments of neurons and variations in fiber tract connections, vertebrate brains are commonly thought to share a basic set of genes. While this may indeed be the case, most gene expression data has focused on mammals and is frequently limited to examination of adult animals in a relatively few species.

Non-mammalian amniotes, reptiles and birds, have brains that differ significantly from those of even the most primitive mammals [1]. This is particularly true of the forebrain. Despite these differences, early development of one forebrain part, the diencephalon, is similar in species as diverse as: mouse [2], chick [3-5], and *Alligator* [6]. Accordingly, later development is inferred to be the period when differences occur.

Expression of certain genes was investigated during a time period towards the end of development in a representative reptile, *Alligator mississipiensis*. The goal of these experiments was to provide an initial set of data that could be compared with other amniotes. Genes investigated were chosen because they were considered to be: essential for cell function (voltage-gated sodium channel isoforms, NaV1.1 and NaV1.2); involved in synapse formation (synaptophysin and synaptic vesicle 2a [SV2a]); or a member of the calcium binding protein family (calbindin 2). In addition, the choice of two of these genes, calbindin 2 and SV2a, was influenced by prior experiments using immunocytochemistry and Western blot techniques that identified calbindin in the midbrain and forebrain in juvenile crocodilians [7] and *Alligator* embryos [8] and by immunocytochemistry experiments that have demonstrated SV2a in the forebrain in developing *Alligator* embryos (Pritz, unpublished observations).

With regard to the expression of these genes, two questions were asked. First, are these genes expressed differently in the common brain divisions: telencephalon; diencephalon; midbrain; and hindbrain? Second, do changes in expression of these genes occur over time? Although limited, these observations provide the first analysis of quantitative mRNA expression in any reptilian brain during development and will form the basis for further and more detailed analyses.

2. Materials and Methods

2.1. Specimens

Alligator mississipiensis eggs were obtained from the Rockefeller Wildlife Refuge in Grand Chenier, Louisiana, and were housed in an incubator at 30°C. Embryos were removed from eggs and staged. Five brains were used at the following stages: stage 22.5 (N=2); stage 24 (N-1); and stage 24.5 (N=2). After staging, animals were euthanized by occipito-cervical transection. Brains were placed in a solution of 5% glycerol and artificial cerebrospinal fluid and stored at –80°C until tissue was processed. Brains were divided into four parts: telencephalon; diencephalon; midbrain; and hindbrain (see Figure 1). The diencephalon included the

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thalamus and hypothalamus and the cerebellum was included as part of the hindbrain.

2.2. Quantitative RT-PCR

Methodology used here has been described previously [9,10]. Briefly, total RNA was isolated from the lysates of *Alligator* embryos via the RNeasy Mini Kit for the extraction of RNA from tissue (Qiagen) and treated with TURBO DNAse (Ambion) and EDTA. Single-stranded cDNA was synthesized using reverse transcriptase (Bioline) and Oligo-dT primers. The cDNA was amplified for quantitative RT-PCR with the SYBR Green PCR Master Mix (Applied Biosystems) on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). A mixture of cDNA template, SYBR Green Master Mix, and forward and reverse primers (see Table 1) was treated with uracil N-glycosylase before undergoing the following protocol: 1×, 95°C, 10 min; 45×, 95°C, 15 sec, 60°C, 1 min; 1×, 95°C, 15 sec, 60°C, 15 sec, 95°C, 15 sec (for melting curve analysis); 72°C, hold. The PCR products were analyzed with the ABI PRISM sequence detection software (Applied Biosystems). Resulting mRNA levels were compared to L27, a ribosomal housekeeping gene. The mRNA level for each probe (x) relative to L27 mRNA (internal control) was calculated as follows: $\text{mRNA}(x\%) = 2^{Ct\,(L27)-Ct(x)} \times 100$.

3. Results

Messenger RNA (mRNA) expression of five genes was assessed in *Alligator* embryos. Results are presented by gene type, brain region, and developmental stage.

3.1. NaV1.1

Expression of this gene varied according to brain region: highest in the hindbrain and then progressively diminishing as one advanced rostrally towards the telencephalon (Figure 2A). Over time, expression in the diencephalon decreased while that in the telencephalon increased. However, in neither case were these changes significant (Figure 2B, C).

3.2. NaV1.2

Depending on brain region, gene expression varied. Its pattern (Figure 3A) clearly differed from that of NaV1.1 (Figure 2A). The greatest relative mRNA expression was in the midbrain followed by the hindbrain, diencephalon, and telencephalon (Figure 3A). With regard to the diencephalon and telencephalon, no significant change occurred over time (Figure 3B, C).

3.3. SV2a

SV2a expression in the diencephalon and telencephalon was markedly more robust than that of any of the genes investigated. Although no appreciable change occurred in the diencephalon over time, a significant increase occurred in the telencephalon (Figure 4).

3.4. Synaptophysin

Because synaptophysin was not expressed in the diencephalon, data on this gene were available only for the telencephalon (Figure 5). Over time, mRNA expression increased, although this change did not reach statistical significance.

3.5. Calbindin

Calbindin gene expression in the diencephalon was greater than that observed in the telencephalon (Figure 6). Expression in the diencephalon did not change over time while that in the telencephalon decreased. However, none of these changes reached statistical significance (Figure 6).

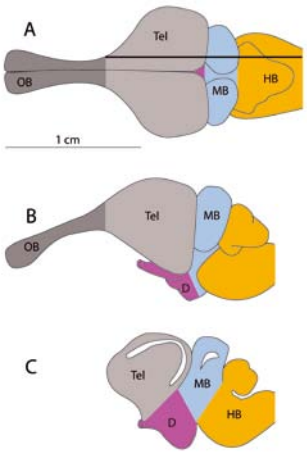

Figure 1. Schematic outline of an *Alligator* brain. Dorsal (A), lateral (B), and parasagittal views are illustrated. The solid line in the dorsal view (A) illustrates the location of the parasagittal view (C). Similar color-coded areas represent similar brain divisions. Because some morphological changes occur in *Alligator* brains over the stages investigated, primarily affecting olfactory bulb size and shape, a more generalized schematic is shown to illustrate the approximate location of brain divisions sampled. Abbreviations: D, diencephalon; HB, hindbrain; MB, midbrain; OB, olfactory bulb; Tel, telencephalon.

Table 1. Primers used for amplification of various genes from *Alligator* embryos.

Gene	Forward primer	Reverse primer	Product size (bp)
NaV1.1	AGAGGGAAGTTGGGATTGATG	TGGTGATTTGGAACAGGCAG	77
NaV1.2	GTTTTCCTCTCCACACCAGTC	AACCAATATCCTTCACCCGAC	112
Synaptophysin	GCAGTGTTCGCTTTCATGTG	GTTCCTTGCATGTGTTTCCTG	139
Synaptic Vesicle 2a (SV2a)	GGTTCCATCCCCATAGTCTTC	CATCCAGAACATACAGAGCCAG	93
Calbindin 2	ATGAGAATGAACTGGACGCC	CCTTTCTGTAGAGCTTCCCTG	131
L27	GGTCATCGTGAAGAACATTG	CATGGCAGCTGTCACTTTC	103

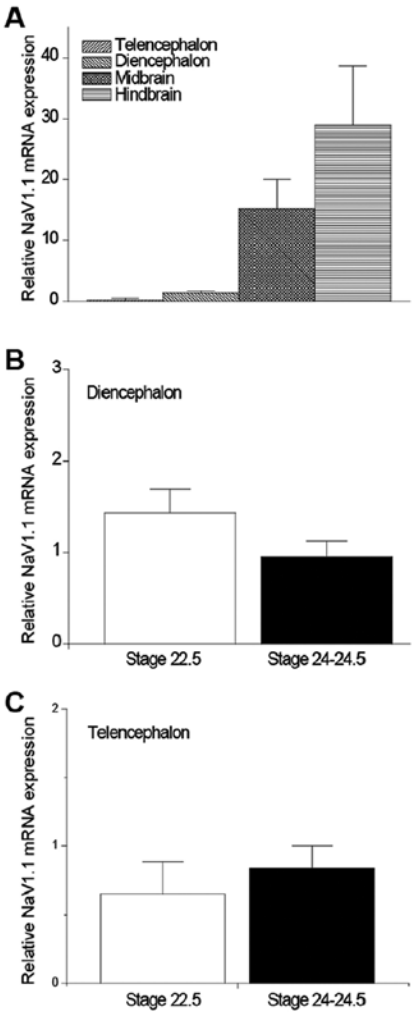

Figure 2. Quantitative RT-PCR for NaV1.1 gene from various regions and stages of *Alligator* embryos. (A) mRNA for Na+ channel isoform NaV1.1 was detected in the telencephalon, diencephalon, midbrain and hindbrain. Expression of NaV1.1 mRNA was significantly higher in the midbrain and hindbrain when compared with the diencephalon and telencephalon. The NaV1.1 mRNA level was not different between stage 22.5 and stage 24-24.5 for either the diencephalon (B) or telencephalon (C). For figures 2-6, data were expressed as fold change over expression of L27 mRNA (a ribosomal internal control gene) ± SEM. For each stage examined, the reaction was run in triplicate. Accordingly, for stage 22.5, N=6 and for stages 24 and 24.5 that were grouped together, N =9.

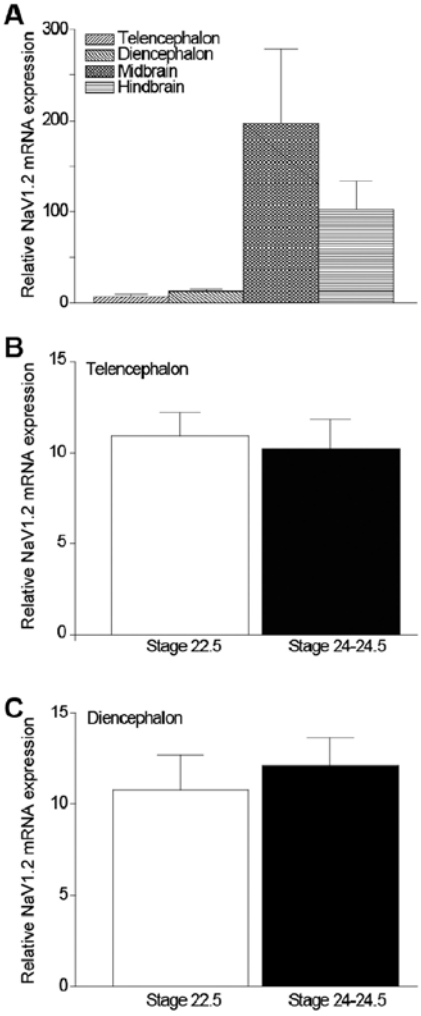

Figure 3. Quantitative RT-PCR for NaV1.2 gene from various regions and stages of *Alligator* embryos. (A) mRNA for Na+ channel isoform NaV1.2 was detected in the telencephalon, diencephalon, midbrain and hindbrain. Expression of NaV1.2 mRNA was significantly higher in the midbrain and hindbrain when compared with the diencephalon and telencephalon. The NaV1.2 mRNA level was not different between stage 22.5 and stage 24-24.5 for either the diencephalon (C) or telencephalon (B).

4. Discussion

Measures were undertaken to standardize tissue from the brain regions being sampled. For this reason, the olfactory bulbs were excluded. In the diencephalon, the rostral and caudal borders were based on an oblique plane that was consistent through samples. A similar situation applied to the midbrain where the caudal boundary was the isthmus. In the hindbrain, a similar caudal extent minimizing any spinal cord inclusion was attempted. Second, by its very nature, analyzed tissue contained more than neurons. Other components, such as glia and blood vessels, all formed part of the brain regions being analyzed. However, other data based on immunocytochemical observations [7,8] (also, Pritz, unpublished observations) indicated that at least for SV2 and calbindin, a portion of the expression observed was clearly neuronal in origin.

In *Alligator*, development occurs over a period of approximately 65 days before hatching and is divided into 28 stages according to morphological features [11]. In the hindbrain, transformation into segments known as rhombomeres is complete by stage 11.5 [12]. In the diencephalon, major subdivisions, prosomeres, are finalized by stage 14.5. Thereafter, internal cytoarchitectonic regions become recognizable [6]. Thus, the stages examined in this analysis occurred relatively late in development.

The expression of the sodium channel alpha isoforms, NaV1.1 and NaV1.2, varied according to brain region and, for the data available, did not parallel each other. Encoded by the gene *SCN1A*, NaV1.1 is primarily expressed in neuronal cell bodies [13] and correlates with the rising phase of the action potential. In mammalian brains, NaV1.1 expression has been reported in neurons of the deep cerebellar nuclei and somato-dendritic regions of Purkinje cells, suggesting that these isoforms are involved in the integration of synaptic input. Encoded by the gene *SCN2A*, NaV1.2, is expressed mainly in cerebellar granule cell parallel fibers [13]. Despite the limited region and developmental stage analyses reported here, the expression of the different isoforms varied by 8- to 10-fold, regardless of region or developmental stage (Figures 2 and 3). These findings are remarkably consistent with a 7-fold change reported in the mRNAs for the two isoforms in human brain [14]. The expression of Nav1.2 was also reported to be much higher than Nav1.1 in the hippocampus and cerebral cortex in rodents [15]. This remarkable conservation of the relative ratio of NaV1.1 and NaV1.2 between *Alligator*, rodents, and humans may reflect their distinct roles and relative importance in the brain.

Synaptic vesicle protein 2a (SV2a) is an integral membrane glycoprotein present in all

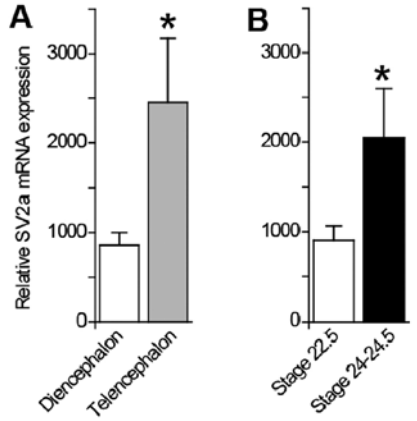

Figure 4. Quantitative RT-PCR for SV2a gene from the forebrain of *Alligator* embryos at various developmental stages. (A) mRNA for SV2a was significantly higher in the telencephalon when compared with the diencephalon (*, p <0.05, Students' t-test). (B) Expression of SV2a mRNA was significantly higher in the forebrain (telencephalon and diencephalon combined) at stage 24-24.5 when compared with stage 22.5 (*, p <0.05, Students' t-test).

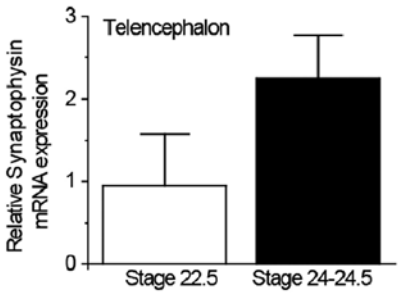

Figure 5. Quantitative RT-PCR for synaptophysin gene from the telencephalon of *Alligator* embryos. mRNA for synaptophysin was not significantly different in the telencephalon between stage 24-24.5 when compared with stage 22.5 (p>0.05, Students' t-test).

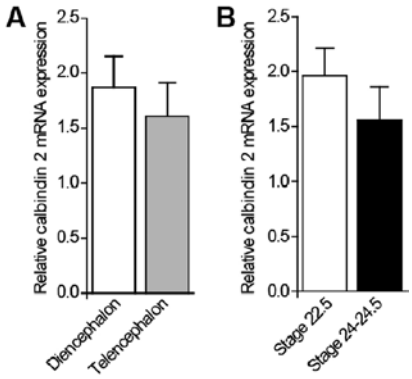

Figure 6. Quantitative RT-PCR for calbindin 2 gene from the forebrain of *Alligator* embryos at various developmental stages. (A) mRNA for calbindin 2 was not different in the telencephalon when compared with the diencephalon (p>0.05, Students' t-test). (B) Expression of calbindin 2 mRNA was also not different in the forebrain (telencephalon and diencephalon combined) between stage 24-24.5 and stage 22.5 (p>0.05, Students' t-test).

synaptic vesicles and regulates the expression and trafficking of the calcium sensor protein synaptotagmin and contributes to transmitter release [16]. SV2a is expressed ubiquitously throughout the brain with the highest levels in subcortical regions [17]. In chick, SV2a is present in presynaptic terminals of climbing and mossy fibers and in the inner molecular layer of the cerebellum during late development in rodents [18]. SV2a expression in the telencephalon not only showed a dramatic increase over time but also was far more robustly expressed than in the diencephalon. This increase presumably reflects an increase in synapse formation that occurs during development. Current immunocytochemical studies on SV2 (Pritz, unpublished observations) as well as ongoing studies examining the formation of thalamo-telencephalic connections in *Alligator* (Pritz, unpublished observations) should help to clarify the significance of this increase in SV2a gene expression.

Synaptophysin is the major integral membrane glycoprotein of synaptic vesicles and has been used as a marker of brain synapses. Although abundant, the role of this protein remains unclear. Synaptophysin may be important for regulating the kinetics of synaptic vesicle endocytosis in neurons [19]. Synaptophysin mRNA is abundantly distributed throughout all major brain regions and was detectable as early as embryonic day 14 in rodent brains, which precedes synaptogenesis [19]. An explanation for the expression of synaptophysin in the telencephalon but not the diencephalon during the time intervals examined remains unclear.

While preliminary, calbindin gene expression data did not change significantly over the time interval examined nor did it vary between the diencephalon and telencephalon. These data will be correlated with ongoing experiments investigating calbindin immunocytochemical expression during these time periods.

The present observations provide preliminary quantitative data on the expression of five genes in several brain regions over a restricted time period towards the end of development in *Alligator*. Some of these observations will be correlated with ongoing experiments as noted above. Furthermore, the deciphering of the genome of *Alligator* is presently underway. These future data will help in the analysis of the present gene expression observations. The data reported here as well as the ongoing experiments noted above should provide a more complete picture of the expression of these genes in different brain regions in *Alligator* over time. When more information becomes available, these data can be compared with observations on birds and mammals to explain how similarities and differences in gene expression produced different amniote brains.

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