SELECTIVE MOLECULAR MODIFICATION TO CALCIUM CONDUCTANCE DOMAINS OF NMDA RECEPTOR GLUN2 SUBUNITS REGULATES MATURATION OF HIPPOCAMPAL BEHAVIORS

by

Erin Morgan Sanders
A Dissertation
Submitted to the
Graduate Faculty
of
George Mason University
in Partial Fulfillment of
The Requirements for the Degree
of
Doctor of Philosophy
Neuroscience

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Date: ____________________________  Summer Semester 2014
George Mason University
Fairfax, VA
Selective Molecular Modification to Calcium Conductance Domains of NMDA Receptor GluN2 Subunits Regulates Maturation of Hippocampal Behavior

A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at George Mason University

by

Erin Morgan Sanders
Doctor of Science Philosophy
George Mason University, 1987

Director: Theodore C. Dumas, Assistant Professor
Department of Neuroscience

Summer Semester 2014
George Mason University
Fairfax, VA
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DEDICATION

I would like to dedicate this dissertation to my ohana. ‘Ohana’ is Hawaiian for ‘family’ and I have been so fortunate to be surrounded by such supportive people in my home, my dojo, and my lab. I could not have done this without any of you.
ACKNOWLEDGEMENTS

I would like to acknowledge all the people in academia who have helped instill not only the attributes of diligence necessary for research but also the amazingly rewarding desire to teach and mentor. Drs. Lou and Karen Burnette and Dr. Ed Wirth with the College of Charleston, thank-you for mentoring me in my first molecular investigations in your summer program for research experience for undergraduates (REU). My life-long dream to become a marine biologist had finally become realized. I would like to thank Dr. Nora Terwilliger and Dr. John Postlethwait, at the University of Oregon, for further enriching my career in marine research and teaching experience at both elementary school and college level students. They gave me the opportunity to become interested in development which became the focus of my Masters research while I taught science to both elementary school and college students. It was while immersing myself in both the classroom and at the bench that I became acutely aware of my awakened interest in cognitive development. Almost serendipitously, I was offered the opportunity to come to George Mason University by my advisor, Dr. Ted Dumas to investigate and generate a novel animal model for autism. Since being in the PBNJ lab, I have met many people on the spectrum and family members of these people and it has been a huge driving force to help me persist in research that I find to be both compelling and vital for better understanding something we know so little truth about and that affects so many people.

I would also like to especially thank my advisor, Dr. Ted Dumas, and my committee members, Dr. Daniel Cox, Dr. John Cressman, and Dr. Raja Parasuraman for all their support and critiques of my research, dissertation and publications. I have been fortunate to grow as a scientist and contribute to scientific research by the opportunities afforded to me by the Krasnow Institute for Advanced Learning at George Mason University.
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LIST OF ABBREVIATIONS

Transgenic Line with GluN2A Amino/TMD and GluN2B Carboxy Regions .......... ABc
Adult ................................................................. Ad
α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor .................. AMPAR
Transgenic Line with GluN2B Amino/TMD and GluN2A Carboxy Regions ........ BAc
Base Pairs ......................................................... BP
Cornu Ammonis One .......................................................... CA1
4',6-diamidino-2-phenylindole ............................................................... DAPI
Dentate Gyrus ................................................................. DG
Di-Heteromeric Receptor ................................................................. DI
ES(E/D)V Carboxy terminus sequence ....................................................... ESDV
N-methyl-D-aspartate Receptor Subunit 1 ............................................... GluN1
N-methyl-D-aspartate Receptor Subunit 2 ............................................... GluN2
N-methyl-D-aspartate Receptor Subunit 2A ............................................. GluN2A
N-methyl-D-aspartate Receptor Subunit 2B ............................................. GluN2B
Hemagglutinin ................................................................. HA
Honest Significant Difference ............................................................. HSD
Immunohistochemistry ................................................................. IHC
Immediate ................................................................. IMM
In Situ Hybridization ................................................................. ISH
Moleculare ................................................................. MOL
N-methyl-D-aspartate receptor ............................................................. NMDAR
Postnatal Day ................................................................. P
Phosphate Buffered Solution ............................................................... PBS
Polymerase Chain Reaction ............................................................... PCR
Paraformaldehyde ................................................................. PFA
Postsynaptic Density ................................................................. PSD
Postsynaptic Density Protein 95 ........................................................... PSD95
Radiatum ................................................................. RAD
Room Temperature ................................................................. RT
Spontaneous Alternation ................................................................. SA
Synapse-Associated Protein 102 ............................................................ SAP102
Tetracycline Response Element ........................................................... TRE
Tri-Heteromeric Receptor ................................................................. TRI
Tetracycline Transactivator ................................................................. tTA
Transmembrane Domain ................................................................. TMD
Wildtype ................................................................. WT

x
ABSTRACT

SELECTIVE MOLECULAR MODIFICATION TO CALCIUM CONDUCTANCE DOMAINS OF NMDA RECEPTOR GLUN2 SUBUNITS REGULATES MATURATION OF HIPPOCAMPAL BEHAVIOR

Erin Morgan Sanders, Ph.D.

George Mason University, 2014

Dissertation Director: Dr. Theodore C. Dumas

The hippocampus is essential for formation and retrieval of autobiographical memories, deliberative decision-making, and spatial navigation. One emerging model to better understand detailed relationships between hippocampal activity and complex cognitive abilities is to investigate neural modifications during the postnatal period when hippocampal-dependent behaviors are first displayed. Electrophysiological and behavioral indices of contextual encoding in rodents are present at roughly seventeen days of age, while spatial navigation is delayed until the end of the third postnatal week. Given younger animals seem capable of encoding space, why does spatial navigation require nearly a week more of development to emerge? Modifications in glutamate receptors occur in parallel with the emergence of spatial navigation. Prominently, N-methyl-D-aspartate receptors (NMDARs) containing GluN2B are replaced by NMDARs with GluN2A subunits during the third postnatal week. This subunit switch produces
alterations in calcium conductance profiles and intracellular protein-protein signaling of NMDARs, both of which are implicated in activity-dependent synaptic plasticity and likely regulate the developmental emergence of spatial navigation. We engineered transgenic mice to express chimeric GluN2 subunits to isolate amino acid domains regulating calcium conduction from those dictating intracellular protein-protein signaling. We documented expression of chimeric GluN2 subunits and applied behavioral assays at three postnatal weeks of age. Results indicate a primary role for alterations in calcium conductance in the emergence of spatial navigation. These findings define molecular and physiological factors that are critical for spatial navigation and provide basic information about neural and cognitive development that also help to understand neurodevelopmental disorders.
INTRODUCTION

Autobiographical memories allow us to relate past experiences to current situations, to plan future scenarios, to create narratives and, in large part, define who we are as individuals. While infants and toddlers rapidly acquire information about their environment and are very good at elemental conditioning, they do not form autobiographical memories in a manner that can be retrieved at older ages. This phase of childhood amnesia terminates at around three years of age (Mullally & Maguire, 2014) as brain systems that encode autobiographical memories mature, namely the hippocampus and surrounding structures. Interdisciplinary investigations on the protracted maturation of the hippocampus in rodents allow for a more detailed understanding of how individual neural processes relate to specific aspects of spatial cognition. For instance, the hippocampus contains all of its post mitotic cells at birth and levels of neurogenesis in the dentate gyrus (DG) are comparable to adults by two weeks of age (Altman et al., 1973; Haggblom et al., 1974; Douglas, 1975; Bayer et al., 1982). Moreover, electrophysiological recording in juvenile rats freely exploring an arena have revealed stable place cells in the hippocampus and grid cells in the medial entorhinal cortex by postnatal day (P) 16 (Wills et al., 2013), suggesting rats possess a structurally mature hippocampus and are capable of contextual encoding by roughly two weeks of age. Interestingly, spatial navigation is not expressed until the end of the third postnatal week.
(Wills et al., 2013), indicating contextual encoding alone is insufficient for spatial navigation. Thus, it is unclear how the hippocampus links contextual encoding to spatial navigation. A better understanding may be gleaned by examining developmental modifications in synaptic transmission that co-occur with the emergence of spatial navigation.

The two main ionotropic receptors at glutamatergic synapses are α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) and NMDARs (Stoneham et al., 2010). AMPARs allow fast depolarization of the postsynaptic neuron in response to presynaptic glutamate release. This initial excitation removes a magnesium block on NMDAR channel pores prolonging the excitatory postsynaptic response and permitting the entry of calcium into the postsynaptic neuron. Depending on the dynamics of calcium entry through NMDARs, AMPARs are subsequently added to or removed from the synapse resulting in long-term potential (LTP) or long-term depression (LTD) of excitatory synaptic efficacy, respectively. LTP and LTD are known to be important for the normal establishment of forebrain neural networks and for information processing and storage (Cline & Haas 2008; Lau & Zukin 2007; Gambrill et al., 2011; Wang & Morris, 2010). Therefore, it is likely that developmental alterations in NMDARs that occur during the third postnatal week are at least partially responsible for the concomitant changes in hippocampal network activity and spatial behavior.

Hippocampal NMDARs are composed of four subunits, two obligatory GluN1 subunits and two auxiliary GluN2 subunits (Monyer et al., 1992). GluN3 subunits are expressed transiently in the hippocampus during early development only (Sanz-Clemente
et al., 2013). GluN2 subunits define numerous functional characteristics of NMDARs including channel dynamics, synaptic anchoring, and intracellular protein-protein signaling (Sanders et al., 2013). Amino terminus (extracellular) and transmembrane domains (TMDs) of GluN2 control receptor assembly as well as channel open probability and deactivation (Sanders et al., 2013). GluN2 carboxy terminus (intracellular) domains contain several amino acid recognition sites for calcium-sensitive molecules such as CAMKII, PP1, and PP2, and other signaling molecules involved in synaptic transmission and plasticity (Sanders et al., 2013). At two weeks of age, synaptic NMDARs in the hippocampus predominantly contain GluN2B subunits, display reduced channel open probability, slower deactivation, and have a higher affinity for CaMKII (Cull-Candy et al., 2001; Dumas, 2005). NMDARs with GluN2B subunits are replaced by NMDARs with GluN2A subunits during the third postnatal week (Dumas, 2005). At this age, NMDARs containing GluN2A subunits display increased open probability, faster channel deactivation, and a reduced affinity for CaMKII (Cull-Candy et al., 2001; Dumas, 2005). Importantly, positioning of NMDARs adjacent to presynaptic release sites is reliant on specific anchoring proteins in the postsynaptic density (Sans et al., 2003; Bessoh et al., 2007). In fact, the GluN2B to GluN2A transition occurs in parallel with a switch from PSD incorporation of SAP102 to PSD95 (Sans et al., 2000; Elias et al., 2008) and genetic deletion of PSD95 prevents the GluN2B to GluN2A transition (Be´i¨que et al., 2006). As such, the GluN2B to GluN2A shift produces a number of functional alterations in NMDARs that potentially regulate hippocampal maturation and that can be studied in isolation through the generation of chimeric GluN2 subunits.
In order to modify individual properties of NMDARs in juvenile mice, we created GluN2 chimeras (Fig. 1). Our “ABc” chimera contains the amino terminus and TMDs of GluN2A fused to the carboxy terminus of GluN2B. In contrast, the “BAc” chimera contains the amino terminus and TMDs of GluN2B fused to the carboxy terminus of GluN2A. Both lines utilize the tetracycline transactivator/tetracycline response element (tTA/TRE) induction system with tTA under transcriptional control of the CaMKII minimal promoter (Mayford et al, 1995) driving expression robustly in hippocampal pyramidal neurons and less so in cortical neurons and hippocampal granule cells beginning around two weeks of age. Thus, in animals just under three weeks of age, having higher GluN2B background expression, the amino terminus and TMDs are selectively altered in ABc mice and the carboxy terminus is selectively altered in BAc mice. In contrast, in animals older than three weeks of age, having higher GluN2A background expression, the amino terminus and TMDs are selectively altered in BAc mice and the carboxy terminus is selectively altered in ABc mice.
This complementary pair of transgenic mouse lines is ideal for investigating the individual contributions of calcium conductance (mediated by the amino and TMDs) and intracellular protein-protein signaling (mediated by the carboxy terminus) (Sanders et al., 2013) to glutamatergic synapse maturation and the developmental emergence of spatial learning and memory. If induction of more mature calcium conductance dynamics is responsible for the emergence of spatial navigation at three weeks of age, ABc animals should display advanced behavioral development. Conversely, if modified intracellular protein-protein signaling is responsible for the emergence of spatial navigation at three weeks of age, BAc animals should display advanced behavioral development. We found

Figure 1. Illustration of chimeric GluN2 subunits. Illustrations of GluN2 chimeras. Hemagglutinin (HA) tags were fused to the amino termini of ABc and BAc.
that juvenile ABc, but not BAc animals, displayed more mature levels of spontaneous alternation (SA) in the Y-maze (Sanders et al., 2013), suggesting that altered calcium conductance produced by the GluN2B to GluN2A shift is a principle factor that limits the final maturation of the hippocampus. In adulthood, ABc animals tested in the Morris water maze (MWM) displayed enhanced spatial memory, while performance of BAc animals was indicative of impaired spatial learning, supporting the idea that combining the GluN2A-type channel properties with GluN2B-type intracellular signaling is optimal for consolidating or retrieving spatial memories. Molecular and histological assays verified transgene expression and translocation into dendrites and revealed an influence of GluN2 chimera expression on levels of synaptic anchoring proteins, SAP102 and PSD95. This study is the first to show a direct influence of developmental alterations in NMDAR composition on hippocampal maturation and supports the need for continued submolecular investigation of NMDAR involvement in neural network construction and information processing and storage. These findings help to elucidate the molecular underpinnings of cognitive development and will enable advancements in treating developmental disorders where early intervention is beneficial.
TRANSGENIC METHODS

Sub-cloning Injection Constructs

We obtained pCIS-A and pCIS-B plasmids with respective mouse Glun2A and Glun2B full-length cDNA (graciously donated by Dr. Ann Stephenson, University College of London). These plasmids served as templates for PCR amplification of fragments to be used to construct the chimera injection constructs. We designed mutagenic forward and reverse primers (capped with restriction sites) to amplify amino terminus + TMD (nucleic acids 1–2481) and carboxy terminus (GluN2A: 2482–4512; GluN2B: 2482–4449) fragments for both genes (Tab. 1). The amino + TMD fragments of GluN2A and GluN2B were separately ligated into pTRE-HA. Subsequently, the carboxy fragment of GluN2A and GluN2B were serially ligated into pTRE-HA-B_{amino+TMD} and pTRE-HA-A_{amino+TMD}, respectively. Ligation was performed by SeqWright and final products were completely sequenced. pTRE-HA-ABc (7265 bp) and pTRE-HA-BAc (7494 bp) injection constructs (Fig. 2) were linearized with Ase1 and Drd1/Fsp1 and sent to the Transgenic Mouse Facility (TMF; University of California, Irvine) where they were injected into fertilized C57BL oocytes that were then implanted into C57BL pseudo-pregnant females. Positive offspring were returned to George Mason University.
Table 1. Primers used to amplify GluN2 fragments for cloning injection construct plasmids.

<table>
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<tr>
<th>Primer &amp; Region</th>
<th>Sequence 5'→3'</th>
<th>Primer Length bp</th>
<th>Size bp</th>
<th>Melting °C</th>
</tr>
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<tr>
<td>ABC F'</td>
<td>AAAAAGTCGACCGGAGACTGGGCTACTGGACCTTTGCTG platform + sal1 + GluN2A (3-28)</td>
<td>39</td>
<td>2500</td>
<td>70.8</td>
</tr>
<tr>
<td>Amino + TM</td>
<td>AAAAAGCGGCAGCGGCAGCCACGTGCTGCTAGAAAACG platform + not1 + kas1 + GluN2A (2457-2475)</td>
<td>38</td>
<td>2500</td>
<td>70.9</td>
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<tr>
<td>ABC F'</td>
<td>AAAAAGCGCCGCCGATGGCAGCTGCTAGAAAACG platform + kas1 + GluN2B (2482-2508)</td>
<td>29</td>
<td>1990</td>
<td>67.2</td>
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<td>Carboxy</td>
<td>GGAAGCGGCCGCCTCAGACATGATCAGACTCAATACGCGCCAGAGGAGTGTG platform + not1 + GluN2B (4417-4449)</td>
<td>32</td>
<td>1990</td>
<td>67</td>
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<tr>
<td>ABC R'</td>
<td>CGCAAAGTCGACGAAAGCCCGCCGAGGAGTGTG platform + sal1 + GluN2B (3-24)</td>
<td>32</td>
<td>2502</td>
<td>70.8</td>
</tr>
<tr>
<td>Amino + TM</td>
<td>AAAAAGCGGCCGCAGCGGCAGCCACATGACATGGGACCTTTGCTG platform + not1 + kas1 + GluN2B (2457-2475)</td>
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<td>2502</td>
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<td>ABC F'</td>
<td>GGTGGCGCCGATGCCCTCAGGCTGCTAGACCTTCATC platform + kas1 + GluN2A (2482-2508)</td>
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<td>2054</td>
<td>71.8</td>
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Transcriptional Regulation of ABc and BAc Transgenes

A total of eleven ABc and nine BAc founder mice in a C57Bl background were returned from UC Irvine. We genotyped these founders to confirm transgenesis and back-crossed them wildtype C57Bl to confirm transmission across generations. For genotyping, we performed PCR on genomic DNA (isolated from tail snips) and used primers we designed to amplify the HA-amino region of GluN2A or B (Tab. 2 and Fig. 3). The complementary touchdown region of the forward genotyping primer sits in the TRE sequence, which is unique to the transgene. Positive founders (and subsequently their offspring) were bred with line tTA-84 (donated from Dr. Cliff Kentros, University of Oregon) and line tTA-Mayford breeders (obtained from Jackson Labs). These mice express tTA under transcriptional control of the CaMKII minimal promoter (Mayford et al., 1995) and have different patterns of expression in the forebrain. In both tTA lines, expression is heavy in hippocampal pyramidal neurons. Line tTA-84 shows weaker expression in the neocortex and minimal expression in the DG and striatum (Results, Fig.

Figure 2. Linearized ABc and BAc injection constructs. Over-loaded linearized A)ABc and B)BAc injection constructs (boxed in red). C)Digest details.
6. Line tTA-Mayford expresses tTA more heavily in the neocortex, DG, and striatum (Results, Fig. 6). Primers for genotyping tTA animals were supplied by Dr. Clifford Kentros (Tab. 2 and Fig. 3). Due to apparent false positive identification of ABc and BAc mice with the chimera genotyping primers, all GluN2 and tTA genotyping was confirmed commercially (Transnetyx). In an unfortunate DNA recombination evident, tTA-Mayford and ABc are always expressed together or not at all. All data below are collapsed across tTA lines.
**Table 2. GluN2 genotyping primers.** We designed primers to amplify GluN2A and GluN2B amino sequence downstream from TRE. Dr. Cliff Kentros donated primers for amplifying tTA.

<table>
<thead>
<tr>
<th>Primer &amp; Region</th>
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<th>Product Size bp</th>
<th>Melting °C</th>
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<tr>
<td>TRE509 F'</td>
<td>CCCATACGATGGTTCCAG pTRE-HA (509-525)</td>
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<tr>
<td>ABc Amino R'</td>
<td>GCGTTATTGATGAACCG GluN2A (1 - 228)</td>
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<td>44.6</td>
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<tr>
<td>BAc Amino R'</td>
<td>GGGCCATCCATTGAACAG GluN2B (1 – 456)</td>
<td>511</td>
<td>50</td>
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<tr>
<td>tTA-O-F 5'</td>
<td>AGGTCGAGGAATTCAACAGG</td>
<td>387</td>
<td>58</td>
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<td>tTA-O-R 5'</td>
<td>ACAATCTTGGCTCACTGCAA</td>
<td>387</td>
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<tr>
<td>tTA-I-R 5'</td>
<td>CGTCGTCGTCCTTGTAGTCG</td>
<td>300</td>
<td>58</td>
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<tr>
<td>tTA-I-F 5'</td>
<td>GTACGAGACCCTGGAGAACC</td>
<td>300</td>
<td>58</td>
</tr>
</tbody>
</table>
Figure 3. PCR reactions with genotyping primers for ABc and BAc transgenic lines. Genomic DNA from tail preps of ABc and BAc animals were used as template for genotyping PCR. **Top row:** ABc (A) and BAc (B) tailpreps genotyped with tTA primers. **Bottom row:** ABc (C) and BAc (D) tailpreps genotyped with GluN2 primers. Boxed samples are from ABc (red) and BAc (yellow) transgenic animals expressing chimeric GluN2 subunits.
CHIMERA EXPRESSION

In Situ Hybridization (ISH)

Riboprobe Generation

Several riboprobes were generated to identify chimeric GluN2 mRNA by excising sequences from pTRE-HA (ABc) or pTRE-HA (BAc) and ligating into Bluescript (pBSK-KS+, Stratagene), which contains start sites for commercially available RNA polymerases. Initial long riboprobes (750 bp, Fig. 4) targeted the HA and amino regions of the ABc and BAc mRNA. We observed positive labeling in ABc, BAc, and WT tissue sections indicating non-specificity of the probes (Results Figure 7; top). Given this result, we designed and generated a second set of riboprobe templates to produce 81 bp riboprobes (Fig. 5) (ABc antisense sequence: 5’ GUA GCC CAG UCU GCC CAU GGU CGA CCA AGC UUG GGC CUC CAU GGC CAU AAG AGC GUA AUC UGG AAC AUC GUA UGG GUA CAU 3’ – BAc antisense sequence: 5’CUC UGC GCU GGG CUU CAU GGU CGA CCA AGC UUG GGC CUC CAU GGC CAU AAG AGC GUA AUC UGG AAC AUC GUA UGG GUA CAU 3’). Shortening the riboprobes increased the proportion that was unique to the transgenic mRNA. The 81 bp riboprobes produced
positive signal in ABc and BAc but not WT sections with a high signal-to-noise ratio (Results Fig. 7; bottom).

Figure 4. Generating ABc and BAc 750 bp riboprobes. Lanes: 1) Fermentas 1kb ladder 2) Cla1 linearized A750-pBSK 3) Cla1 linearized B750-pBSK 4) ABc and 5) BAc antisense RNA (riboprobe).
At P17-19 and 1-2 months of age, animals were anesthetized with Isoflurane and perfused with 4% paraformaldehyde (PFA). Brains were removed and stored in PFA for 6 hours and then switched to 30% sucrose. Cryopreserved brains were sectioned on a cryostat at 30 µm thickness and stored on slides at -70°C. *In vitro* transcription reactions to produce riboprobe were carried out at 37°C for two hours. Single-stranded riboprobe products were visualized by agarose electrophoresis through 1xMOPS/DEPC-treated gel (0.75% for 750 bp probes or 2% for 81 bp probes). Riboprobe was diluted in
hybridization buffer (Sigma; PerfectHyb™ Plus) and denatured at 70°C. Denatured probe was applied to pre-warmed tissue section and incubated overnight (62°C). The following day, excess probe was removed with SSC-(50%) formamide and MABT washes. Tissue was then blocked for an hour at room temperature (RT) and incubated overnight at room temperature (RT) with anti-dig antibody (1:1500). Excess antibody was removed with MABT and Tris-HCL (pH 9.5) washes and tissue was then transferred to 10 mM Levamisol, 3.5 µl/ml NBT, and 2.6µl/ml BCIP and incubated at 37°C for 4 hours to develop the colorimetric reaction. Afterwards, the color reaction was stopped by washing with PBS-T (RT) and tissue sections were dehydrated through ascending alcohol concentrations, defatted with xylenes, and coverslipped. Bright field images were captured at 2-20X magnification (Fig. 6).

ISH Results

Images in Figure 6 are of P17 and adult tTA-84, tTA-Mayford, and WT sagittal sections reacted with a tTA riboprobe. Both tTA lines show dense tTA expression in hippocampal pyramidal cells in area CA1. The tTA-Mayford line also expresses heavily in areas CA2, CA3, the DG, neocortex, dorsal thalamus, and dorsal striatum. Line tTA-84 expresses lightly in hippocampal regions outside of area CA1 (CA2, CA3, DG) and in the neocortex. Images in Figure 7 are of the hippocampus in adult ABc_tTA-Mayford,
BAc_tTA-Mayford, and WT sagittal sections reacted with ABc or BAc riboprobe. ABc and BAc expression in these crosses are evenly distributed across excitatory neurons throughout the hippocampus.

**Figure 6. ISH with tTA riboprobe.** Sagittal hemi-sections (30μm) showing tTA expression in P17 (left) and adult (right) tTA-84 (top), tTA Mayford (middle), and Wildtype (bottom) mice.
Western Blot

Methods

Adult (1-2 months) animals were anesthetized with Isoflurane and decapitated. Neocortex and hippocampi were dissected and homogenized in a 0.32M sucrose homogenization buffer according to Al Hallaq et al. (2007). After lysis with water and protease inhibitors, membranes were sedimented by centrifugation (25,000 g, 30 min, 4°C) and re-suspended in minimal homogenization buffer before storage at -70°C. Total synaptic protein was quantified using the RC/DC Protein Assay Kit (Bio-Rad). Protein samples (25 µg) were separated by (7%) SDS PAGE and transferred to nitrocellulose membranes (0.45 µm) using a Mini-Trans Blot (Bio-Rad). Non-specific binding was
prevented by blocking in PBS with 0.1% i-Block powder. Primary antibody incubation continued overnight (1:200 dilution, 4°C). Secondary antibody was applied for 2 hr (1:2500 dilution, room temp). Blots were visualized with BCIP and NBT (in 100 mM Tris-HCl, pH 9.5). Antibodies include α-HA (rabbit monoclonal), α-GluN2A (rabbit and goat polyclonal), α-actin (mouse monoclonal), α-rabbit IgG (AP-conjugated), and α-mouse IgG (AP-conjugated), all acquired from Millipore and Santa Cruz Biotechnologies. Blots were imaged on Syngene GBox which allowed us to analyze band size and intensity compared to ladder samples with known protein sizes and quantities.

**Results**

We were able to demonstrate GluN2A (~170 kda) and HA expression alongside an actin control (~43 kda) in membrane fractions of neocortex and hippocampus homogenates from adult ABc (Mayford) mice (Fig. 8). Initial band quantification on blots yielded inconsistent results, most likely due to differences in final re-suspension and subsequent quantification of protein. Our lab currently uses the (BioRad) Quick Start Bradford Dye kit, which generally seems to yield more consistent results. GluN2 chimera protein analysis moved forward with immunohistochemistry to investigate anatomical specificity in ABc and BAc mice across development.
Immunohistochemistry (IHC)

Methods

IHC was performed to ensure that chimeric GluN2 NMDARs were translocated to dendrites. Brains from ABc, BAc, and WT animals at P17-19 and 1-2 months of age were harvested and cryopreserved as stated for in situ hybridization. Sagittal sections were cut on a vibratome at 30 µm and processed in a free-floating IHC protocol. Slices were permeabilized with 0.1% triton-X and pretreated with Na borohydride (2ng/ml) before washing in PBS and blocking with normal goat serum (1%) for one hour. Sections were washed again in PBS and incubated for 72 hours in 1:450 primary antibodies at 4°C.
Anti-HA (rabbit polyclonal, Rockland) was co-applied with α-PSD95 (mouse monoclonal; Thermo Fisher Scientific) or α-SAP102 (mouse monoclonal; Rockland) primary antibodies. Some sections did not receive primary antibody. Slices were subsequently washed with PBS and incubated for 4 hours at RT with 1:500 α-rabbit-fluorescein (green) and α-mouse-Cy3 (red) secondary antibodies. After final PBS washes, slices were mounted on glass slides and coverslipped in Vectashield with or without DAPI. Whole hippocampi were imaged on a compound microscope (20x) (Experiment 1, Fig. 9). HA expression in area CA1 was imaged confocally at 40X in sections from P17 and adult animals (Experiment 2, Fig. 10). For additional double-labeling experiments, images of the stratum radiatum were captured at 20X, 40X, and 60X magnification (Experiment 3, Fig. 11). Microscope settings were adjusted to minimize negative control intensities and signal intensities were normalized to background regions within sections. HA intensities (like synaptic anchoring signal intensities) were normalized to intensities from control regions within each section (using Photoshop and Metamorph) and are presented as a ratio of WT intensities (Fig. 12).

Results

Experiment 1: HA and GluN2A co-localization

Initial IHC experiments used the same α-HA (mouse monoclonal; Millipore) and α-GluN2A (rabbit-goat polyclonal; Millipore) primary antibodies from our Western blot experiments and did not include DAPI-counterstaining. We successfully demonstrated colocalization of HA and GluN2A (Fig. 9) in the radiatum, moleculare, and the DG.
moleculare. More specifically, Both HA (red) and GluN2A (green) signals were present and colocalized in sections from ABc mice, resulting in yellow hue in the dendrites through the hippocampus. Only GluN2A (green) was observed in WT sections. Due to unacceptable levels of nonspecific labeling with this particular HA antibody during replication, subsequent IHC experiments utilized primary antibodies optimized for immunohistochemistry protocols.

**Figure 9. Immunohistochemical labeling for GluN2A and HA in adult mice.** IHC co-labeling (yellow) for GluN2A (green) and HA (red) in A) transgenic ABc (++, top) and B) wildtype (--, bottom) brain sections. Scale bars = 10 μm.

**Experiment 2: HA labeling across development**
Several HA antibodies were tested on ABc, BAc, and WT tissue and an HA antibody from Rockland Inc. was selected to further analyze HA expression across development (Sanders et al., 2013). Figure 10 shows representative results from area CA1 of the hippocampus with pyramidal cell bodies at the top. As judged by HA signal intensities in genetically-positive sections compared to control sections, chimeric ABc and BAc subunits are present in the dendrites of hippocampal pyramidal neurons.

**Figure 10.** Confocal microscope captured immunohistochemical labeling of chimeric GluN2 subunits. HA (green) and DAPI (blue) expression in hippocampal CA1 (40x) cell body layer (top) and stratum radiatum in coronal sections (30 μm).

*Experiment 3: HA co-localization with SAP102 and PSD95 across development*
Confocal images were also collected for HA, SAP102, and PSD95 in sections collected from ABc, BAc, and WT mice at P17 and 1-2 months (adult). Representative images are shown at 20X, 40X, and 60X magnifications (Fig. 11). Yellow color indicating colocalization of HA and PSD95 can be observed at all magnifications. However, distinct green dendrites and spines at 60X suggest at least some ABc subunits are not in the vicinity of PSD95.

![Confocal images](image)

**Figure 11.** Confocal microscope captured immunohistochemical labeling of chimeric GluN2 subunits with DAPI counterstaining. Adult ABc tissue at **A)** 20X, **B)** 40X, and **C)** 60X magnification. Green labeling is HA, Red labeling is PSD95, and blue is DAPI nuclear counterstain.

Signal intensities for HA were compared across age, genotype, and dendritic field. There was a main effect of age on HA signal intensity, reflecting an increase in HA expression with increasing age: \(F(2, 78) = 31.32, P < 0.001\) (Fig. 12). We also found a main effect of genotype \(F(2, 78) = 7.1, p < 0.001\) such that ABc (Tukey HSD, \(p < 0.001\)) and BAc
Tukey HSD, p < 0.006) intensities differed from WT. At P17, there was a main effect of genotype for HA expression in the radiatum [F (2, 11) = 5.8, p < 0.02]. ABc sections had elevated HA signal (Tukey HSD p < 0.02), and BAc sections showed a trend for elevated signal compared to sections from WTs (Tukey HSD, p < 0.07). No significant expression was found at P17 in the molecular layer of area CA1 [F (2, 11) = 1.9, p < 0.19] or the DG [F(2, 11) = 0.61, p < 0.56]. As adults, there was a main effect of genotype on HA expression in the radiatum of area CA1 [F (2, 11) = 8.17, p < 0.007] and the molecular of the DG [F (2, 11) = 14.71, p < 0.001*]. HA signal in ABc (Tukey HSD: radiatum, p < 0.007; DG molecular p < 0.001) and BAc (Tukey HSD: radiatum, p < 0.009; DG molecular p < 0.001) sections was elevated compared to sections from WTs.

There were main effects of age for sections incubated with α-SAP102 [F (1, 48) = 224.65, p < 0.001] or α-PSD95 [F (1, 48) = 140.9, p < 0.001] for all hippocampal regions, reflecting an increase in SAP102 and PSD95 levels from P17 to adulthood. Within the P17 age group, there was a main effect of genotype for SAP102 expression in the radiatum [F (2, 6) = 8.07, p < 0.02]) and molecular in area CA1 [F (2, 6) = 6.28, p < 0.03]. ABc sections showed elevated signal compared to WT in the radiatum (Tukey HSD, p < 0.03) and in the molecular (ABc: Tukey HSD, p < 0.03) but not DG. PSD95 expression at P17 did not differ from wildtype in any region examined. However, in adult sections, there was a main effect of genotype on PSD95 expression in the radiatum [F (2, 6) = 8.61, p < 0.02] and molecular of area CA1 [F (2, 6) = 8.64, p < 0.02] and in the DG [F (2, 6) = 6.1, p < 0.04]. For ABc animals, PSD95 expression was elevated in radiatum (Tukey HSD, p < 0.02) and molecular of area CA1 (Tukey HSD, p < 0.01) and in the
DG (Tukey HSD, p < 0.04). For BAc animals, PSD95 expression was elevated only in the radiatum (Tukey HSD, p < 0.04). Taken together, these IHC results indicate that ABc subunits are expressed at P17 and in adulthood while BAc subunits are expressed at significant levels in adults only (with a trend at P17). Also, increasing chimeric GluN2 expression alters anchoring protein assemblages both at P17 and in adults with a greater impact of ABc subunit expression.
Figure 12. Normalized IHC for HA, SAP102, and PSD95 across development. Immunohistochemistry on P17 and adult (30μm) hippocampal sections co-labeled with HA (top), SAP102 (middle), PSD95 (bottom). Asterisk denotes significance from age-matched WT intensities. Sample size by genotype by antibody: ABc = 3, BAc = 3, WT = 2.
BEHAVIOR

Y-Maze Spontaneous Alternation (SA)

Methods

Naïve animals were tested at P17-19, P22-24, or 1-2 month (Adults). Each animal was placed in the center of the Y-maze (arm length: 32 cm; arm width: 9 cm; wall height: 15 cm) and allowed to freely explore for 8 minutes. The number of arm entries and SA rate were measured for each test. SA is calculated as:

\[
SA = \frac{\text{heterogeneous triads}}{\text{possible triads}}
\]

(triads = three successive arm entries)

(heterogeneous = thee different arm entries in succession)

(possible triads = number of arm entries – 2)

Results

Developmental and genotype effects on Y-maze behavior were found (Fig. 13). Total number of arm entries (Fig. 13A) increased with increasing age [two-way ANOVA, main effect of age, \(F(2,162) = 33.87; \ p < 0.001\)]. There was no main effect of genotype on total number of arm entries. Main effects of age [\(F(2,162) = 4.95; \ p < 0.01\)] and genotype [\(F(2, 162) = 5.31; \ p < 0.01\)] were observed for alternation rate (Fig. 13B). As
previously reported in rats (Dumas, 2004; Blair, Nguyen et al., 2013), alternation rate increased from P17–19 to P22–24 in wildtype mice. Alternation rate in ABc mice was significantly increased at P17–19 compared to age-matched wildtype littermates (Tukey HSD, p < 0.05). At P22-24, alternation rate was decreased in BAc mice compared to age-matched WT controls (p < 0.05). These findings suggest precocious development of SA behavior in ABc mice and delayed development in BAc mice.

Figure 13. Precocious development of spatial exploration with expression of ABc, but not BAc, subunits. (A) Average arm entries are shown for each age and genotype group. (B) Average alternation rates are shown for each age and genotype group. Asterisks indicate significant group differences (Tukey Honest Significant Difference test, P<0.05). Adult animals are 1–2 months of age.
Morris Water Maze (MWM)

Methods

Litters were trained on the spatial version of the Morris Water Maze (MWM). Half of each litter was tested at P17-19 and the entire litter was tested at P22-24, such that half of the animals were tested twice by P22-24. Naïve and previously tested animals were trained as at 1-2 months of age (Adult) as well, however; only data on naïve animals are presented. The testing environment was spatially enriched with various groups of black shapes hanging on a white curtain surrounding two thirds of the pool and an off-white wall and biocontainment hood visible through the curtain gap. The pool diameter was 127 cm and the platform diameter was 17.5 cm. The pool water was made opaque with non-toxic white paint and maintained at a temperature of 24°C.

A one-day massed training protocol was applied to isolate developmental contributions to hippocampal functioning. Just prior to the first trial, animals were pre-trained by being allowed climb onto the platform from three different directions and sit for 10 seconds each time. Immediately following climbing practice, escape training ensued. Escape training consisted of five blocks of three escape trials (15-20 minute inter-block interval) followed by a single probe trial (immediate, IMM), to test spatial learning, and then three more “refresher” escape trials (refresher trials were not included in data analyses). A single long-term memory probe was performed twenty-four hours (24Hr) following training to assess spatial memory. After each training block, animals
were gently towed off and placed in a heated cage (~29°C) until dry and then returned to the home cage to wait for the next trial block. For each trial, the subject was placed at one of three starting positions varied pseudo-randomly along the edge of the pool (starting positions were offset from goal locations by 45°). The escape platform remained at one goal location across all training trials (but shifted for new cohorts) and was positioned one cm below the water surface. Training trials lasted for one minute or until the animal reached the goal platform. If the platform was not found, the animal was guided onto the platform. In both cases, animals remained on the escape platform for fifteen seconds. For probe trials, the platform was removed from the pool and the animal was placed in the pool from one of two furthest start positions and allowed to search for one minute.

Animal position was continually tracked during training and probe trials by an overhead video camera and tracking software (MazeScan, CleverSys Inc., Reston, VA). Escape latencies were extracted from each training trial for all animals and averaged by training block to create learning curves for each animal (Fig. 14-17) and swim velocities were sampled from five animals per group. Search times per quadrant (quadrant dwell time), time searching over goal location, and distance from platform (“directness of initial approach,” 1 Hz sampling rate) were calculated for each probe trial. Two-way repeated-measures ANOVAs (genotype x training block and age x genotype) were used to compare learning curves. Goal quadrant biases for probe trials were calculated within groups by Chi Square test. Time over goal location was analyzed by two-way ANOVA (age x genotype). Directness of initial approach was analyzed by two-way repeated
measures ANOVA (age x genotype x sample time). Tukey HSD post-hoc test was used to determine individual group differences.

Results

Escape Training: Wildtype across age groups
Natural developmental changes in spatial learning and memory were first analyzed in WT animals. Repeated measures ANOVA analyses of escape latencies during training revealed main effects of age \( F(2, 192) = 58.21; p < 0.001 \) and training block \( F(4, 721) = 59.46, p < 0.001 \). In agreement with prior work (Paylor et al., 1996), WT animals tested at P22-24 (Tukey HSD, p<0.001) and as adults (1-2 months; Tukey HSD, p<0.001) displayed reduced escape latencies compared to animals tested at P17-19 (Fig. 14A). There was no main effect of age (Fig. 14B) \( F(2, 15) = 2.247, p<.140 \) or training block \( F(4, 60) = 0.81, p < 0.52 \) on swim velocity. Combined, these results suggest improved escape learning from P17-19 to P22-24.
Escape Training: P17-19 across genotypes

Each age group was examined individually to assess genotype effects on escape training with two-way repeated measures ANOVA. At P17-19, while there was no main effect of genotype \( [F(2, 99) = 1.17, p < 0.32] \) (Fig. 15A), escape latencies did decrease across training blocks \( [F(4, 382) = 7.51, p < 0.001] \). Furthermore, when only the first three blocks were analyzed, a main effect of genotype emerged \( [F(2, 99) = 3.26, p < 0.04] \). ABc animals required less time to find the escape platform than age-matched WT controls (Tukey HSD, \( p < 0.05 \)). Combined, the data suggest a trend for more rapid

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**Figure 14:** WT escape learning across age groups. A) Escape learning curves: significant age effect \( (p< 0.001) \) for learning by P22-24 (Tukey HSD \( P<0.001 \)). B) Swim speed: no age effect. Sample size by age group for escape latency: P17-19 = 45, P22-24 = 74, and Adults = 76. Sample size by age group for swim speed: P17-19 = 5, P22-24 = 5, and Adults = 8.
learning in ABc animals at P17-19 with no difference in end performance. At this age, there was no main effect of genotype on swim speed [F (2, 12) = 0.421, p<.666], however, velocity did decrease across training blocks [F (4, 48) = 7.5, p < 0.001] (Fig. 15B).

![Graph A and B showing time and velocity across blocks for genotypes ABc, BAc, and WT.]

**Figure 15: P17-19 escape learning across genotypes. A)** Escape learning curves: no genotype effect. Axis is scaled to emphasize increased ABc performance in block 1-3. **B)** Swim speed: no genotype effect. Sample size by genotype for escape latency: ABc =33, BAc = 24, and WT = 45. Sample size by genotype for swim speed: ABc = 5, BAc = 5, and WT = 5.

*Escape Training: P22-24 across genotypes*

Although a two-way repeated measures ANOVA run at P22-24 revealed no main effect of genotype [F (2, 67) = 0.96, p < 0.39] (Fig. 16A), escape latencies decreased across
training blocks \( [F (4, 243) = 8.75, p < 0.001] \). Swim velocity decreased across training blocks for all genotypes \( [F (4, 48) = 7.88, p < 0.001] \) and there was a main effect of genotype \( [F (2, 12) = 3.982, p< 0.05] \). ABc animals showed a trend for swimming slower compared to WT (Tukey HSD, \( p< 0.09 \)) (Fig. 16B).

**Figure 16: P22-24 escape learning across genotypes. A)** Escape learning curves: no genotype effect. **B)** Swim speed: no genotype effect. Sample size by genotype for escape latency: ABc = 18, BAc = 12, and WT = 40. Sample size by genotype for swim speed: ABc = 5, BAc = 5, and WT = 5.

**Escape Training: Adult animals across genotypes**

For adult animals, a two-way repeated measures ANOVA revealed no main effect of genotype \( [F (2, 73) =2.1, p < 0.14] \) although escape latencies decreased across training blocks \( [F (4, 258) = 25.9, p < 0.001] \) (Fig. 17A). When analyzed for only the first three
blocks, a main effect of genotype emerged \( [F (2, 73) = 3.9, p < 0.02] \). Escape latencies were greater for BAc mice compared to WTs (Tukey HSD, \( p < 0.02 \)). Swim speed analysis revealed no main effect of genotype \( [F (2, 15) = 0.016, p < 0.984] \) although velocities decreased across training blocks \( [F (4, 48) = 7.9, p < 0.001] \) (Fig. 17B). Combined, the data suggest a trend for slower learning in adult BAc animals compared to WTs.

**Figure 17: Adult escape learning across genotypes.** A) Escape learning: no genotype effect. B) Swim speed: no genotype effect. Sample size by genotype for escape latency: ABc = 25, BAc = 14, and WT = 37. Sample size by genotype for swim speed: ABc = 5, BAc = 5, and WT = 8.

*Spatial Learning: Wildtype across age groups*
We first assessed performance parameters for the immediate (IMM) probe in naïve wildtype animals across development. The traditional measure of dwell time in each quadrant of the testing pool revealed a goal quadrant bias at P22-24 ($\chi^2 = 9.92; p < 0.02$) and in adults ($\chi^2 = 3.77; p < 0.005$), but not at P17-19 (Fig. 18A). To further assess spatial strategy, one-way ANOVAs of time spent directly over goal location showed a significant main effect of age [$F (2, 107) = 14.2, p < 0.001$] (Fig. 18B). Post hoc analyses indicated that adult animals (Tukey HSD, $p < 0.001$) and P22-24 animals (Tukey HSD, $p < 0.006$) spent more time over the goal location than animals tested at P17-19. Swim speed analysis revealed a main effect of age [$F (2, 118) = 22.62 p < 0.001$] where P22-24 (Tukey HSD, $p < 0.001$) and adults (Tukey HSD, $p < 0.001$) swam faster compared to P17-19 animals (Fig. 18C). Distance to the goal location was measured for each second of the probe for the first 10 seconds to assess directness of initial approach. This analysis found a main effect of age [$F (2, 221) = 23.9, p < 0.001$] where initial approach was less direct at P17-19 versus P22-24 (Tukey HSD $p < 0.001$) or adult animals (Tukey HSD $p < 0.001$) (Fig. 18D). Initial approach was not different between animals tested at P22-24 and in adulthood (Tukey HSD $p < 0.30$). Overall, these data demonstrate that spatial learning reaches adult levels in WTs by P22-24.
Spatial Learning: P17-19 across genotypes

At P17-19, no significant quadrant bias were revealed by Chi Square analyses for ABc ($\chi^2 = 7.5; p < 0.1$), BAc ($\chi^2 = 7.7; p < 0.1$), and WT ($\chi^2 = 7.3; p < 0.1$) (Fig. 19A). Likewise, there was no genotype effect [$F (2, 83) = 0.134, p < 0.88$] for time spent over the goal location (Fig. 19B), for swim speed [$F (2, 94) = 0.918, p < 0.4$] (Fig. 19C), or for
The directness of initial approach \( F(2, 101) = 1.51, p < 0.226 \) (Fig. 19D). These results suggest no effect of ABc or BAc expression on spatial learning in animals under three weeks of age.

**Figure 19.** P17-19 spatial learning across genotypes. **A)** Quadrant dwell times: no bias. **B)** Time over goal location: no genotype effect. **C)** Swim velocity: no genotype effect. **D)** Directness of initial approach (first 10 seconds) to goal location: no genotype effect. Sample size by genotype: ABc = 28, BAc = 21, and WT = 37.

**Spatial Learning:** P22-24 across genotypes
At P22-24, only WT animals displayed a goal quadrant bias ($\chi^2 = 9.92; p < 0.02$) ($\chi^2 = 9.92; p < 0.02$) (Fig. 20A). Likewise, there was no genotype effect [$F(2, 66) = 0.1, p < 0.91$] for time spent over the goal location (Fig. 20B), for swim speed [$F(2, 66) = 0.15, p < 0.87$] (Fig. 20C), or for directness of initial approach [$F(2, 65) = 2.25, p < 0.11$] (Fig. 20D). However, if the distance to platform analysis is extended to 30 seconds, to assay *persistence* of search, a significant genotype effect emerges [$F(2, 65) = 3.5, p < 0.04$]. BAc (Tukey HSD, $p < 0.03$) animals are less persistent in using a direct spatial search strategy to find the platform location than WTs.
Spatial Learning: Adults across genotypes

By adulthood, WT and ABc animals showed significant goal quadrant biases (WT: $\chi^2 = 13.76, p < 0.005$; ABc: $\chi^2 = 11.96, p < 0.02$) while BAc ($\chi^2 = 3.3; p < 0.25$) showed no bias (Fig. 21A). Time spent over the goal location revealed a genotype effect [$F(2, 63) = 3.78; p < 0.03$] where BAc animals spent less time compared to WT animals (Tukey HSD $p < 0.05$) (Fig. 21B). There was no genotype effect for swim speed [$F(2, 72) = 0.06; p < 0.94$] (Fig. 21C), ANOVA analyses of directness of initial approach toward the goal
location revealed a significant effect of genotype \([F (2, 66) = 3.54, p < 0.04]\) where BAc animals searched less directly compared to WT animals (Tukey HSD \(p < 0.03\)) (Fig. 21D). Thus, by adulthood, ABc and WT mice display similar levels of spatial learning, but BAc animals are impaired.

**Figure 21. Adult spatial learning across genotypes.** A) Quadrant dwell times: WT \((\chi^2 = 13.76; p<0.005)\) and ABc \((\chi^2 = 11.96; p < 0.02)\) significant bias. B) Time over goal location: significant genotype effect \((p=0.05)\). C) Swim velocity: no genotype effect D) Directness of initial approach (first 10 seconds) to goal location: significant genotype effect \((p=0.05)\). Sample size by genotype: ABc = 25, BAc = 12, and Adults = 29.
**Spatial memory: Wildtype across age groups**

To assess spatial memory, we first looked at dwell time in each quadrant of the testing pool during the 24-hour probe trial. No significant Chi Square tests for quadrant dwell time were observed at P17-19 ($\chi^2 = 7.3; p < 0.1$) or P22-24 ($\chi^2 = 7.7; p < 0.1$). Adult mice did show quadrant bias ($\chi^2 = 13.56, p < 0.005$) (Fig. 22A). Moreover, there was an age effect on time over goal location [$F(2, 110) = 12.89, p < 0.001$] where P22-24 (Tukey HSD, $p < 0.006$) and adults (Tukey HSD, $p < 0.001$) spent more time over the goal location compared to P17-19 (Fig. 22B). A similar significant age effect was seen in swim speed [$F(2, 116) = 13.88, p < 0.001$] where P22-24 (Tukey HSD, $p < 0.001$) and adults (Tukey HSD, $p < 0.001$) had faster velocities (Fig. 22C). Repeated measures ANOVA for the distance to platform during the first ten seconds of the probe trial revealed a significant age effect [$F(2, 221) = 14.97, p < 0.001$]. Approach was more direct at P22-24 (Tukey HSD, $p < 0.02$) and as adults (Tukey HSD, $p < 0.001$) compared to P17-19 (Fig. 22D). Combined, the data support the idea that spatial memory tested twenty-four hours after training matures between P17-19 and P22-24.
At P17-19, as revealed by Chi Square values, no goal quadrant biases were observed for ABc ($\chi^2 = 7.5; p < 0.1$), BAc ($\chi^2 = 7.7; p < 0.1$), or WT ($\chi^2 = 7.3; p < 0.1$) (Fig. 23A). Despite no quadrant bias, there was a significant genotype effect [$F (2, 75) = 4.5, p < 0.01$] for time spent over the goal location where ABc (Tukey HSD, $p < 0.01$) spent more time over goal location compared to WT (Fig. 23B). This effect was not a result of...
genotypic differences in swim speed \( F (2, 94) = 1.27, p < 0.29 \) (Fig. 23C). A significant effect of genotype was found for directness of initial approach \( F (2, 81) = 3.62, p < 0.03 \) (Fig. 23D). ABc animals approached more directly than WTs (Tukey HSD, \( p < 0.05 \)). These results support the notion that, while performance overall is poor, some degree of improved spatial memory consolidation or retrieval is observed in ABc mice under three weeks of age.
Spatial memory: P22-24 across genotypes

At P22-24, only naïve wildtypes showed a significant Chi Square result ($\chi^2 = 8.73$, $p < 0.05$), though the shape of the distribution did not indicate a goal quadrant bias (Fig. 24A). There was also no genotype effect for time over goal location [$F (2, 65) = 1.39; p < 0.255$] (Fig. 24B), swim speed [$F (2, 64) = 0.17; p < 0.85$] (Fig. 24C), or directness in
initial approach to goal location [F (2, 60) = 1.6; p<.212] (Fig. 24D). Thus, according to these measures, there was no effect of genotype on spatial memory at this developmental stage.

**Figure 24. P22-24 spatial memory across genotypes.** A) Quadrant dwell times: WT ($\chi^2 = 8.73, p < 0.05$) significant bias. B) Time over the goal location: no genotype effect. C) Swim velocity: no genotype effect. D) Directness of initial approach (first 10 seconds) to goal location: no genotype effect. Sample size by genotype: ABc = 18, BAc = 12, WT = 38.

**Spatial memory: Adults across genotypes**

Only adult WT animals displayed a significant quadrant bias ($\chi^2 = 8.73, p < 0.05$) (Fig. 25A). Analyzing time over goal location revealed a significant genotype effect [F (2, 60) = 3.22, p < 0.05] where BAc mice spent less time over the goal location (Tukey HSD, p <
0.04) compared to WT (Fig. 25B). There was no genotype effect on swim speed [F (2, 69) = 0.21; p < 0.81] (Fig. 25C). There was a significant genotype effect on directness of initial approach [F (2, 71) = 6.24, p < 0.003], where ABc animals were more direct compared to WT (Tukey HSD, p < 0.03) and BAc (Tukey HSD, p <0.004) (Fig. 25D). Taken together, ABc mice show improved spatial memory compared to WTs, while in BAc mice, a spatial memory deficit is observed secondary to a spatial learning impairment.
Figure 25. Adult spatial memory across genotypes. A) Quadrant dwell times: WT ($\chi^2 = 8.73$, p < 0.05) significant bias. B) Time over goal location: significant genotype effect (p < 0.04). C) Swim velocity: no genotype effect. D) Directness of initial approach (first 10 seconds) to goal location: significant genotype effect (p < 0.006). Sample size by genotype: ABc = 23, BAc = 18, WT = 35.
DISCUSSION

We have successfully generated the first lines of transgenic mice that express chimeric NMDAR subunits in the forebrain for the study of cognitive ability. We confirmed genomic integration and transmission across generations through PCR amplification of the transgene from genomic DNA (Fig. 3) and verified mRNA production in cortical and hippocampal neurons through ISH (Fig. 7). ABc and BAc mRNA were expressed in all hippocampal subregions and to a lesser degree in the neocortex. Chimeric protein translation and translocation to neuronal dendrites was revealed by IHC (Fig. 11). ABc was present as significant levels in the stratum radiatum at P17. In adulthood, ABc and BAc were present in the radiatum of CA1 and the molecular of the DG. SAP102 levels were elevated in ABc mice at P17-19 in the radiatum and molecular of area CA1, while adult ABc and BAc mice showed elevated SAP102 in the radiatum. As adults, both lines displayed elevated levels of SAP102 compared to WTs in stratum radiatum (Fig. 12). Levels of PSD95 were unaltered at P17 but were increased across regions in adult ABc and BAc mice. Developmental increases in SAP102 and PSD95 could result from increased synaptic content of these proteins or an increase in the total number of synapses. Mismatch between changes in SAP102 and PSD95 across postnatal ages supports the former idea, that synaptic content of PSD anchoring proteins is affected by ABc or BAc expression. However, this question is
better addressed through electrophysiological recording, which will be performed in future studies of these chimeric GluN2 lines. Different patterns of alteration in fluorescent signal between chimeric subunits (green channel) and PSD anchoring proteins (red channel) support the idea that changes in anchoring protein levels were not an artifact of excitation/emission bleed through across channels but instead reflected differences in the expression levels of chimeric GluN2 subunits and PSD anchoring proteins (i.e. signal levels are due to biological variables and not technical anomalies).

Regulation of hippocampal-dependent spatial navigation by GluN2 subunits during the late postnatal period was first investigated by testing juvenile and young adult ABc, BAc, and WT mice in a Y-maze. In WT animals, spontaneous alteration in the Y-maze increased from P17-19 to P22-24, corroborating prior work (Douglas et al., 1973, Dumas, 2004). Interestingly, ABc mice alternated at increased rates compared to age-matched WT littermates at P17-19, while BAc mice tended to be delayed in development of alternation rate (Sanders et al., 2013). By young adulthood, there was no difference across genotype groups in alternation rate indicating that selective modification to GluN2 subunit domains that regulate calcium conductance influences navigation behavior in the Y-maze only in juvenile animals. These findings suggest that the endogenous GluN2B to GluN2A subunit switch influences hippocampal-dependent behavior by altering calcium conductance through NMDARs. Selective effect in the ABc, and not the BAc line, at P17-19 supports the idea that the behavioral modification was due to modification of the amino acid sequence of GluN2 subunits and not a non-specific effect of transgenic overexpression. However, due to the distributed forebrain expression patterns produced
by the tTA driver lines, it is not possible to definitively ascribe changes in behavior to expression of ABc or BAc within the hippocampus, though prior lesion studies (Douglas & Isaacson, 1965) suggest that this is the most likely explanation.

Developmental MWM performance in our WT animals is in agreement with prior work in rats and mice (Rudy et al; 1987; Paylor et al., 1996). Substantial improvements in spatial learning (Fig. 16) and memory (Fig. 22) were observed from P17-19 to P22-24, with minimal increases from P22-24 to adulthood. At all tested ages, there was no main effect of genotype for escape learning across all training blocks (Fig. 15, 16, 17). At P17-19, there was no main effect of genotype on spatial learning in the IMM probe (Fig. 19). At P22-24, BAc animals were less persistent in searching for the goal location (Fig. 20). Analysis of the 24Hr probe revealed more considerable effects of genetic manipulation on spatial memory. ABc displayed numerous measures of improved spatial memory at P17-19 (Fig. 23) and in adulthood (Fig. 25). As adults, BAc animals showed impaired spatial learning (Fig. 21) and spatial memory (Fig. 25), indicated by less time spent over the platform location as well as a less direct approach to the goal location on the 24Hr probe (Fig. 25). These findings suggest that GluN2A-type calcium conductance is important for the developmental emergence of spatial learning. Spatial memory appears to be optimized when a GluN2A-type calcium conductance is combined with GluN2B-dependent intracellular signaling. These results provide the first direct evidence that the late postnatal GluN2 subunit switch regulates the postnatal age at which spatial learning and memory are first observed.
The late postnatal emergence of hippocampal-dependent cognitive abilities is thought to result from adjustments in the induction properties for activity-dependent synaptic plasticity (Dumas, 2005a; Dumas, 2005b). Indeed, the threshold for induction of long-term potentiation (LTP) declines and the induction threshold for long-term depression (LTD) increases with increasing age beginning at two postnatal weeks (Dumas, 2012). Since postysnaptic calcium entry during patterned stimulation is known to be a key determinant in the magnitude and direction of change in synaptic efficacy (Bear and Malenka, 2004), it is likely that the modifications to NMDAR calcium conductance dynamics that result from the GluN2B to GluN2A switch are related to the late postnatal adjustments in induction thresholds for LTP and LTD. GluN2-dependent alterations in plasticity induction thresholds have been demonstrated in adults (reviewed in Dumas, 2005). However, this is an open question that can be addressed directly through electrophysiological recording of synaptic responses in hippocampal slices prepared from ABc and BAc mice at varying stages of development. If the shifts in the

### Table 3. Summary of behavior for ABc and BAc across development.

<table>
<thead>
<tr>
<th>Spontaneous Alternation</th>
<th>Escape Latency</th>
<th>Quadrant Bias</th>
<th>Time Over Platform</th>
<th>Direct Approach</th>
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<td>ABc</td>
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<td>↑ Spatial Memory @ P17-19, P22-24 &amp; Adults vs WT</td>
</tr>
<tr>
<td>BAc</td>
<td>No Difference</td>
<td>No significant bias at any age</td>
<td>↓ Spatial Learning &amp; Memory in Adults vs WT</td>
<td>↓ Spatial Learning in Adults vs WT</td>
</tr>
<tr>
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<td>Learning by P22-24</td>
<td>Learning by P22-24</td>
<td>Significant bias by P22-24</td>
<td>Learning &amp; Memory by P22-24</td>
</tr>
</tbody>
</table>


induction thresholds for LTP and LTD occurred at an earlier age in ABc mice, it would
support the involvement of alterations in NMDAR calcium conductance dynamics. It
would then be necessary to identify the change in channel open probability or response
decay rate as the primary biophysical mechanism.

Molecular studies also implicate the GluN2B carboxy terminus in recruitment of
plasticity-related proteins to synapses during plasticity induction. Biochemical assays
have demonstrated a higher affinity of CaMKII for GluN2B than GluN2A (Strack et al.,
2000) and mutations to the CaMKII binding domain in the carboxy terminus of GluN2B
blocks LTP induction (Barria & Malinow, 2005). Interestingly, at one week of age, when
GluN2B content in synaptic NMDARs is high, hippocampal LTP is not reliant on
CaMKII, but rather is dependent on protein kinase A (Yasuda et al., 2003). CaMKII is
not required for LTP induction until after three postnatal weeks of age. Therefore,
because GluN2A content in NMDARs and CaMKII influence over LTP induction both
increase with increasing age, the logic that GluN2B subunits promote synaptic plasticity
by recruiting CaMKII is not so straightforward.

More recent studies involving expression of GluN2 subunits with carboxy
terminus deletions have shown independent involvement of the carboxy tails of GluN2A
and GluN2B in LTP induction (Kohr et al., 2003; Barria & Malinow; 2005; Berberich et
al., 2007; Foster et al., 2010, Wang et al., 2011), In a complementary manner, our
findings emphasize the impacts of calcium conductance domains in NMDAR function.
Thus, in opposition to the notion that GluN2A and GluN2B regulate LTP and LTD,
respectively (Liu et al., 2004; Massey et al., 2004; Izumi et al., 2006), it appears more
likely that individual functional domains of GluN2A and GluN2B act together to optimize plasticity induction. Specifically, the combination of GluN2A calcium conductance dynamics combined with intracellular GluN2B carboxy recruitment of critical plasticity proteins (CaMKII) would be predicted to produce the lowest threshold for LTP induction at mature synapses. This perspective also highlights the need for future studies to focus on the involvement of tri-heteromeric NMDARs in synaptic plasticity induction.

Triheteromeric NMDARs contain one GluN2A, one GluN2B, and two GluN1 subunits and display calcium conductance properties that are intermediate to di-heteromeric NMDARs with GluN2A and GluN2B (Rauner & Korh, 2011). Prior studies point to higher proportions of tri-heteromeric NMDARs at mature versus less mature glutamatergic synapses in the hippocampus (Al-Hallaq et al., 2007; Rauner & Korh, 2011; Wang et al., 2011), though developmental changes in synaptic content of triheteromeric NMDARs have not been studied explicitly. To consider how triheteromerics play a role in our transgenic lines, we made a diagram to propose GluN2B and GluN2A contributions with and without tri-heteromeric receptors (Fig. 26). Assuming complete transformation where both native GluN2 subunits are replaced by chimeric GluN2 subunits within a receptor, the molecular outcome is the same for synapses with diheteromeric or triheteromeric NMDARs. The situation becomes more complicated if only one subunit is replaced. Assuming diheteromeric NMDARs, ABc mice under three weeks of age may have NMDARs with both A- and B-type amino terminus and TMDs (ABc and native BBc) while BAc mice over three weeks of age may
have NMDARs with both A- and B-type animo terminus and TMDs (BAc and native AAc). Conversely, BAc mice under three weeks of age may have NMDARs with both A- and B-type carboxy termini (BAc and native BBc) while ABc mice over three weeks of age may have NMDARs with both A- and B-type carboxy termini (ABc and native AAc). This might be viewed as equivalent to half of the genetic dosage when only one native subunit is replaced by a chimeric subunit. The situation is one step more complicated for triheteromeric NMDARs because the chimeric subunit may replace either the GluN2A or GluN2B subunit. Native triheteromeric NMDARs are 50% GluN2A and 50% GluN2B. If ABc subunits selectively replace GluN2B subunits, the A-type amino terminus and TMD representation increases. If ABc subunits selectively replace GluN2A subunits, the B-type carboxy terminus representation increases. The inverse scenario occurs with single subunit BAc replacement in a native triheteromeric background. If BAc subunits selectively replace GluN2B subunits, the A-type carboxy terminus representation increases. If BAc subunits selectively replace GluN2A subunits, the B-type amino terminus and TMD representation increases.
With the most simple transformation scheme in mind, two subunit replacement per NMDAR, ABc mice likely display increased spontaneous alternation and enhanced spatial memory at P17-19 compared to WTs because they have more NMDARs with GluN2A-type calcium conductance properties. In contrast, BAc mice likely display a tendency for reduced alternation rates and impaired spatial learning and memory at P22-.
24 and older because they have fewer NMDARs with GluN2A-type calcium conductance properties. ABc mice may show superior memory to WTs as adults because they have NMDARs that conduct calcium with an A phenotype but recruit plasticity proteins to synapses with a B phenotype. However, our immunohistochemical data that indirectly suggest a possible increase in synapse number of synapses in adulthood (Fig. 12) warrant further histological and electrophysiological assessment of alterations in synapses number in ABc and BAc mice. Alternatively, a change in the total number of NMDARs at hippocampal synapses could explain the behavioral alterations and should be clarified.

In summary, the current studies indicate that manipulations specifically to calcium conductance domains of NMDARs produce significant alterations in hippocampal-dependent behaviors during development and in adulthood. The findings support further submolecular analyses and interdisciplinary assessment of NMDAR function across the lifespan and inform the creation of improved treatments for developmental and adult disorders where NMDARs are implicated, including Fragile X syndrome, autism spectrum disorders, and schizophrenia.
BACKGROUND

Developmental Trajectories of Learning and Memory Systems Highlighting the Hippocampus

Behavior studies indicate that, across the spectrum of sensory and spatial conditioning tests, short-term memory processes appear before long-term memory processes (Dumas, 2005b). Memory savings lasting hours can be observed during the first postnatal week (Hoffman & Spear, 1988; Cheatle & Rudy, 1979; Stehouwer and Campbell, 1980; Miller et al., 1989), by the second postnatal week, memory savings can be observed 24-48hrs later (Kenny & Blass, 1977; Markiewicz et al., 1986) and by the end of the third postnatal week, memories lasting more than one week can be observed (Campbell & Campbell, 1962; Rudy, 1993; Rudy and Morledge, 1994). In some cases, memory duration increases until P54 and remains retrievable for at least 42 days (Campbell & Campbell, 1962). A similar parallel can be observed in humans with a phenomenon known as “childhood amnesia” which describes the later emergence of stable long-term episodic memory savings (Mullally & Maguire, 2014). Despite classical conditioning experiments demonstrating that rodents can make many meaningful associations early in development, spatial navigation does not emerge until the end of the third postnatal week (Dumas, 2005b). The delay for higher associative processes suggests
that elemental behavioral systems and interconnections between systems must be present before more complex cognitive functions can be expressed (Stanton, 2000). Supportive of this idea, juvenile rodents share similar behaviors as adult rodents with hippocampal lesions (Kurz et al., 1984; Campbell et al., 1969; Feigley et al., 1972) (Tab. 4). However, treatment with an allosteric modulator of AMPARs only thirty minutes before Y-maze testing leads to higher spontaneous alternation rates at P17-19 providing strong support for complete structural maturation and interconnection of the hippocampus at P17-19 but a persistent deficit in excitatory synapse function (Blair et al., 2013).

### Table 4. Cognitive parallels between intact juvenile rats and adults with hippocampal lesions.

<table>
<thead>
<tr>
<th>Proficient</th>
<th>Impaired</th>
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<tbody>
<tr>
<td>Passive Avoidance</td>
<td>Spatial Navigation</td>
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<tr>
<td>Olfactory Conditioning</td>
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**GluN2 structure and forebrain synapse development**

Like all NMDAR subunits, GluN2A and GluN2B subunits consist of three basic parts, an extracellular amino region, a series of four transmembrane (TM) segments with connecting extracellular and intracellular loops, and an intracellular carboxy terminus (Fig. 27). Briefly, the extracellular amino region contains an amino terminal domain (ATD) that is sensitive to small molecule modulation and regulates channel open
probability and deactivation (Gielen et al., 2009; Yuan et al., 2009) as well as an S1S2 “Venus flytrap” ligand binding domain (LBD) for glutamate (Paoletti and Neyton, 2007). In addition to forming the ion pore, the TM series has signaling sequences that participate in subunit assembly (Cao et al., 2011; Salussolia et al., 2011) and N-glycosylation sites in the extracellular loop domain that confer either constitutive or activity-dependent synaptic delivery (Storey et al., 2011). The intracellular carboxy terminus contains various sequences that act as sites for interaction with anchoring and signaling proteins in the postsynaptic density (PSD). Well described signaling domains in the carboxy terminus include binding and phosphorylation sites for alpha calcium-calmodulin kinase type-2 (αCaMKII), a mediator of activity-dependent long-term potentiation (LTP) and long-term depression (LTD) of synaptic efficacy (Merrill et al., 2005), phosphorylation sites for the tyrosine kinases, Src and Fyn, that regulate channel function (Sala and Sheng, 1999), an adaptor protein 2 (AP-2) binding site that regulates receptor internalization (YEKL, Lavezzari et al., 2004; Prybylowski et al., 2005), and a proximal ESDV sequence that permits anchoring at PDZ domains of scaffolding proteins (e.g. membrane-associated guanylate kinase proteins, MAGUKs) in the PSD (Sheng, 2001) (Fig. 2). The most proximal ESDV signal has been described as a peg that fits into a PSD anchoring “slot” (Newpher and Ehlers, 2009). Due to postnatal modifications in the types of anchoring proteins that occupy the PSD, this slot and peg phenomenon has important ramifications for developmental regulation of synaptic NMDAR composition as well as proteomic analysis of NMDAR molecular complexes (Husi et al., 2000) and the design of genetically modified mice with altered GluN2 subunit expression.
<table>
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<th>Domain/ Moif</th>
<th>AA Number</th>
<th>Function</th>
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<td>(823-1063)</td>
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</table>

**Figure 27. GluN2 functional domain signaling sites.**

Chart of known signaling sites for GluN2A and GluN2B. Sites are separated by major segment (amino in white, transmembrane in light gray, or carboxy in dark gray). Bold text indicates a subunit selective interaction.
During postnatal development of forebrain synapses, NMDARs transform from containing GluN2B to GluN2A subunits (Fig. 3, “Wildtype”). This switch occurs in different forebrain regions at different ages (Crair and Malenka, 1995; Nase et al., 1999; Quinlan et al., 1999; Lu et al., 2001; Hsieh et al., 2002), is sensitive to changes in neuronal activity (Bellone and Nicoll, 2007), and is likely regulated at numerous steps of the NMDAR lifecycle (Yashiro and Philpot, 2008). Increasing postnatal age and neuronal activity are associated with increased GluN2A expression (Monyer et al., 1994; Cull-Candy et al., 2001) and synaptic delivery relative to GluN2B (Barria and Malinow, 2002; Storey et al., 2011), reduced GluN2B translation (Chen and Bear, 2006), and increased susceptibility of GluN2B to internalization (Sanz-Clemente et al., 2010) and ubiquitin-dependent degradation (Jurd et al., 2007). All of these factors favor GluN2A availability at the synapse. Expression appears to be a key regulatory step because the relative levels of GluN2A and GluN2B at synapses can be partially offset by transgenic overexpression of GluN2 subunits, as seen in cultured cerebellar granule cells (Prybylowski et al., 2005), organotypic hippocampal slices (Gambrill and Barria, 2011), and in the hippocampus in vivo (Tang et al., 1999, Wang et al., 2009). However, these factors do not dictate the specific synaptic localization of newly arriving NMDARs either to be near neurotransmitter release sites or to reside perisynaptically.

Importantly, specific placement of NMDARs, i.e. synaptic or perisynaptic, can alter receptor function due to distance from transmitter release sites (Kullmann and Asztely, 1998) and interaction with different intracellular signaling streams (Hardingham et al., 2002; Ehlers, 2003; Ivanov et al., 2006). While NMDARs have been reported to be
synaptic or perisynaptic, developmental replacement of GluN2B by GluN2A at hippocampal synapses (Barria and Malinow, 2002) is dependent on the match between the cytoplasmic carboxy terminal domain of GluN2 and the predominant MAGUK expressed at a given postnatal age (Sheng, 2001; Kohr, 2006). For instance, during early postnatal development, immature hippocampal synapses contain SAP102 and NMDARs with GluN2B. During maturation, as SAP102 is replaced by PSD95, NMDARs with GluN2A replace NMDARs with GluN2B (Sans et al., 2000; Elias et al., 2008). The GluN2B to GluN2A shift does not occur in knockout mice that do not express PSD95 (Beique et al., 2006). Functional analyses further suggest that SAP102 associates similarly with GluN2A or GluN2B and that PSD95 selectively associates with GluN2A (Elias et al., 2008), in part through divalent interaction (Bard et al., 2010). Moreover, spontaneous exocytosis of single vesicles primarily activates NMDARs with GluN2A directly beneath transmitter release sites (Townsend et al., 2003; Zhao and Constantine-Paton, 2007), while NMDARs with GluN2B appear more concentrated perisynaptically, as shown by GluN2B pharmacology experiments (Stocca and Vicini, 1998; Dalby and Mody, 2003) and high frequency synaptic activation (Brickley et al., 2003; Lozovaya et al., 2004). Since biochemical assays show that SAP102 and PSD95 can both interact with GluN2A and GluN2B (Al-Hallaq et al., 2007), it appears likely that pre-assembled GluN2A-PSD95 is preferentially inserted at transmitter release sites of more mature animals (Sans et al., 2003; Bessoh et al., 2007). Note that segregated placement is not absolute in that some GluN2B can be found synaptically (Fujisawa and Aoki, 2003; Kohr et al., 2003; Janssen et al., 2005) and some GluN2A can be found perisynaptically (Li et
al, 1998; Mohrmann et al., 2002; Thomas et al., 2006), probably due to a tri-heteromeric composition (i.e. containing GluN1, GluN2A and GluN2B subunits) (Al-Hallaq et al., 2007). In fact, a substantial number of NMDARs at cortical (Sheng et al., 1994; Luo et al., 1997; Kew et al., 1998), cerebellar (Chazot et al., 1994), and hippocampal synapses (Tovar and Westbrook, 1999; Al-Hallaq et al., 2007) appear to contain both GluN2A and GluN2B subunits, and display some functional properties that are intermediate to GluN2A and GluN2B di-heteromeric receptors. Tri-heteromeric NMDARs add further complexity to the study of synaptic NMDAR composition.

The developmental GluN2B to GluN2A subunit switch alters a number of NMDAR properties including affinity for glutamate, small molecules (phenylethanolamines, PEAs) and ions (protons, zinc), channel open probability and deactivation rates, the presence or absence of activity-dependent synaptic integration, and subunit selective interactions with intracellular signaling and anchoring proteins. One or more of these functional properties that are unique to GluN2A or GluN2B are likely responsible for changes in activity-dependent synaptic plasticity that occur late in postnatal development during the GluN2B to GluN2A switch. For instance, prior to the subunit switch, when GluN2B synaptic content is high, NMDAR-dependent LTP at hippocampal synapses is more difficult to induce, but larger in magnitude (Buchanan and Mellor, 2007; Dumas, 2010), and LTD induction is facilitated (Dudek and Bear, 1993). Following the GluN2 subunit switch, it is possible to elicit LTP at lower induction frequencies (Dumas, 2012), and LTD is more difficult to induce (Dudek and Bear, 1993). The GluN2 subunit switch also removes an inhibitory signal that acts to prevent the
insertion of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) into the PSD (Elias et al., 2008; Hall et al., 2007; Adesnik et al., 2008). Removal of this AMPAR inhibitory signal induces silent synapses to become active (Gray et al., 2011) and also alters the threshold for induction of synaptic plasticity (Stoneham et al., 2010). One or more of these developmental modifications in synaptic transmission are likely to be involved in concurrent behavioral changes that are observed in learning and memory tests (Dumas, 2005). Thus, molecular dissection of the functional differences between GluN2A and GluN2B is likely to help explain maturation of glutamate synapses in the forebrain and clarify the involvement of NMDARs in cognitive processes.

**Genetic studies of GluN2**

Much of the evidence for relationships between the GluN2 subunits, synaptic function, and behavior comes from studies that employ molecular and genetic techniques to modify or terminate NMDAR subunit expression. Early studies encountered neonatal lethality with genetic deletion of GluN1 (Li et al., 1994; Forrest et al., 1994), GluN2B (Kutsuwada et al., 1996), or mice lacking only the carboxy terminus of GluN2B (Sprengel et al., 1998), but not following deletion of GluN2A. Initial investigation of adult mice with genetic deletion of GluN2A revealed impaired spatial learning and contextual fear conditioning (Sakimura et al., 1995; Kiyama et al., 1998). LTP was induced at hippocampal synapses only after multiple high frequency bursts were applied, suggesting that LTP could be obtained by activation of NMDARs with GluN2B subunits,
but with a much higher threshold for induction (Sakimura et al., 1995; Kiyama et al., 1998). Combined, these physiological and behavioral results might suggest that GluN2A allows for LTP to be induced more easily or more rapidly, which could explain developmental modifications in LTP induction threshold (Dumas, 2010) and learning and memory abilities (Dumas, 2005). A more recent study employing the same mutant mouse lines lacking GluN2A showed intact spatial reference memory but impaired spatial working memory and impaired single-trial learning (Bannerman et al., 2008). A loss of GluN2A-dependent lowering of LTP threshold might explain the disruption of learning and memory abilities that require more rapid processing, yet maintenance of slower developing reference memory formation in GluN2A knockout mice. Related experiments examined mice expressing truncated GluN2A subunits having no carboxy terminus. Learning and reference memory as well as motor impairments were observed (Sprengel et al., 1998). Furthermore, in the presence of a specific GluN2B antagonist, synaptic NMDAR currents were still present but LTP induction was blocked, indicating that calcium entry alone was insufficient for LTP induction and implicating the C-terminus of GluN2A in LTP induction (Kohr et al., 2003; Berberich et al., 2007). While informative on molecular and physiological levels, these preliminary studies lacked regional anatomical specificity, hindering a greater understanding of brain to behavior relationships, and did not investigate the specific synaptic location of the mutated NMDARs or possible modifications in PSD organization.

Recordings made in hippocampal slices taken from primary GluN2B knockout mice maintained by hand feeding until P2 or P3 revealed impaired induction of LTD
(Kutsuwada et al., 1996). Unfortunately, no assessments of baseline synaptic strength or LTP were made. Using different means to circumvent the lethality produced by GluN2B deletion and increase the age at which recordings can be made, cortical cultures were prepared from late-stage embryonic mice and maintained alive for weeks after preparation (Hall et al., 2007). Obviously, this manipulation precludes behavior testing, but physiological experiments revealed that deletion of GluN2B induced greater surface expression of AMPARs. These findings were corroborated in organotypic slices overexpressing GluN2A or GluN2B (Gambrill and Barria, 2011). While these papers provide a robust description of the mechanisms involved in the GluN2B regulation of AMPAR insertion, no plasticity experiments were performed. More recent GluN2B knock out models, limiting deletion to the late postnatal period and restricted brain structures, further implicated this subunit in functional development of glutamatergic synapses. For instance, mosaic GluN2B deletion from hippocampal neurons enhanced the functional induction of silent synapses during development (Gray et al., 2011). GluN2B deletion limited to cortical and hippocampal pyramidal cells resulted in viable healthy animals (von Engelhardt et al., 2008; Brigman et al., 2010). As adults, these animals performed poorly in spatial learning and memory tests and displayed impaired induction of LTP. Interestingly, when GluN2B was deleted from CA1 pyramidal cells only, animals performed similar to controls in spatial reference memory tasks, but like GluN2A knockouts, were impaired in working memory for recently visited places (von Engelhardt et al., 2008). In both studies, increased afferent stimulation produced LTP at hippocampal synapses, indicating that GluN2B subunits are not absolutely necessary for LTP.
induction. Although a deficit in LTD at hippocampal synapses was reported, a glutamate transporter blocker, which likely increased activation of perisynaptic NMDARs, was necessary for the induction of LTD, confounding interpretations about the function of synaptic NMDARs (Brigman et al., 2010). GluN2A may be preferentially activated in adults due to synaptic positioning closer to neurotransmitter release sites. So, under physiological conditions, it appears that GluN2B-containing NMDARs may play a modulatory role, either by providing greater calcium entry when transmitter release is high or by recruiting intracellular plasticity proteins to the site of calcium entry. The differential impact on rapid versus slower spatial learning in GluN2A or GluN2B knockouts may be a function of the specific neurons that undergo the mutation (i.e. CA1 pyramidal cells). However, GluN1 deletion from CA3 pyramidal cells (Nakazawa et al., 2003) or dentate gyrus granule cells (McHugh et al., 2005) and AMPAR subunit deletion in CA1 pyramidal cells all produce the same behavioral phenotype (Schmitt et al., 2005). So, similarity between the effects of GluN2A and GluN2B deletion could be an artifact resulting from compensatory changes in synaptic organization. More sophisticated mutations to GluN2 subunits, greater neuroanatomical and temporal control over transgene expression, and finer assessment of synaptic placement of mutated subunits will provide greater clarification.

**Next generation genetic studies of the developmental GluN2 switch: Anatomically specific chimeric GluN2 subunit expression in transgenic mice**
The developmental shift in the type of GluN2 subunits found at synapses is mediated by changes in PSD scaffolding proteins (Sheng, 2001). As SAP102 is replaced by PSD95, GluN2B is replaced by GluN2A (Sans et al., 2000; Elias et al., 2008). When GluN2A-containing NMDARs are inserted at synapses, they also displace GluN2B-containing NMDARs perisynaptically (Groc et al., 2009). This information is important not only for understanding synaptic development and plasticity, but also in the design of transgenic mice to study the functional implications of changes in NMDAR composition. That is, the age of the animal, and hence the “context” of the PSD must be taken into consideration (Kohr, 2006). This problem is exemplified in studies investigating the effects of GluN2B overexpression on glutamatergic synaptic transmission in adult visual cortical slices (Philpot et al., 2001) or hippocampal slice cultures after the SAP102 to PSD95 shift (Foster et al., 2010). Negative physiological findings were accompanied by immunolabeling evidence that NMDARs built from transgenic GluN2B subunits do not displace NMDARs with GluN2A, most likely because the adult PSD anchoring composition did not match the transgenic GluN2B carboxy termini of the overexpressed NMDARs.

The ESDV sequence at the tip of the carboxy terminus of the GluN2 subunit interacts directly with PSD anchoring proteins (Gladding and Raymond, 2011). Multiple MAGUKs, including SAP102 and PSD95, interact with GluN2A and GluN2B at this site (Rutter and Stephenson, 2000), suggesting that other direct or indirect subunit-specific interactions exist to confer greater precision placement of GluN2A at synaptic active zones (Cousins et al., 2009), including a divalent structure unique to GluN2A (Bard et al.,
Thus, unless the PSD context is experimentally modified, to transgenically express GluN2 subunits that undergo synaptic insertion, the carboxy terminus must match the type of anchoring protein that predominates at the PSD at the intended age of study. This means overexpressed GluN2B subunits would be inserted near neurotransmitter release sites at immature synapses having a SAP102, but not at mature synapses having a PSD95 phenotype. In contrast, since GluN2A binds similarly with SAP102 and PSD95, overexpressed GluN2A subunits could be inserted near neurotransmitter release sites at immature or mature synapses (Wang et al. 2011). Thus, in contrast to GluN2B overexpression at mature synapses, GluN2A overexpression at immature synapses would be expected to alter NMDAR composition near release sites. Functional changes induced by GluN2A or GluN2B overexpression at mature synapses likely reflect alterations in perisynaptic NMDARs or total numbers of synaptic and perisynaptic receptors.

To more fully understand the effects of the developmental GluN2 subunit switch on synapse composition, synaptic function, and behavior, it is necessary to create genetically modified animals in which the mutation is anatomically restricted to pertinent neurons and is compatible with the composition of the PSD at the age of interest. This requires the generation of chimeric GluN2 subunits in which functional domains and synaptic placement domains are chosen, fused together, and placed under conditional transcriptional control. Some chimeric GluN2 subunits have been generated and used to identify a site in the carboxy tail of GluN2B that induces NMDAR recycling (Tang et al., 2010) and a N-glycosylation site in the extracellular loop between TM3 and TM4 that confers activity-dependency to GluN2A synaptic insertion (Storey et al., 2011). Chimeric
GluN2 constructs have also been expressed in organotypic hippocampal slice cultures. Under these conditions, it was shown that the GluN2B carboxy terminus, but not calcium entering through NMDARs with GluN2B subunits, was essential for LTP induction and the GluN2A carboxy tail served as a dominant negative regulator of LTP induction (Foster et al., 2010). A pre- and postsynaptic pairing protocol was used to slowly induce LTP (in contrast to high frequency induction). Given these caveats, it is possible that the carboxy tail of GluN2B slowly recruits molecules to positions near GluN2A calcium channels to facilitate calcium-induced activation of plasticity-related signaling cascades. Shorter bursts of LTP inducing stimulation are necessary to more closely simulate neuronal activation patterns during sensory stimulation or spatial exploration. Additional chimeras are needed to control synaptic placement of NMDARs having specific GluN2A or GluN2B conductance phenotypes (i.e. open probability, response decay rate, etc.) at immature and mature synapses.
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BIOGRAPHY

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