Differential roles of NMDA receptor subtypes NR2A and NR2B in dendritic branch development and requirement of RasGRF1

Abbreviated title: NR2B-RasGRF1 requirement for dendritic arbor formation

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Abstract

NMDA receptors (NMDARs) are known to regulate axonal refinement and dendritic branching. However, since NMDARs are abundantly present as tri-heteromers (e.g. NR1/NR2A/NR2B) during development, the precise role of the individual subunits NR2A and NR2B in these processes has not been elucidated. Ventral spinal cord neurons (VSCNs) provide a unique opportunity to address this problem, because the expression of both NR2A and NR2B (but not NR1) is down-regulated in culture. Exogenous NR2A or NR2B were introduced into these naturally ‘NR2 null neurons’ at 4 DIV and electrophysiological recordings at 11 DIV confirmed that synaptic NR1NR2A-receptors and NR1NR2B-receptors were formed, respectively. Analysis of the dendritic architecture demonstrated that introduction of NR2B, but not NR2A, dramatically increased the number of secondary and tertiary dendritic branches of VSCNs. Whole-cell patch-clamp recordings further indicated that the newly formed branches in NR2B-expressing neurons were able to establish functional synapses since the frequency of miniature AMPA-receptor synaptic currents was increased. Using previously described mutants, we also found that disruption of the interaction between NR2B and RasGRF1 dramatically impaired dendritic branch formation in VSCNs. The differential role of the NR2A and NR2B subunits and the requirement for RasGRF1 in regulating branch formation was corroborated in hippocampal cultures. We conclude that the association between NR1NR2B-receptors and RasGRF1 is required for dendritic branch formation in VSCNs and hippocampal neurons in vitro. The dominated NR2A expression and the limited interactions of this subunit with the signaling protein RasGRF1 may contribute to the restricted dendritic arbor development in the adult CNS.
Introduction

Dendrites receive most excitatory synaptic inputs and are crucially important for neuronal function and connectivity. Considerable evidence points to a role for the NMDAR in the remodeling of the neuronal architecture during early development (Kalb, 1994; Inglis et al., 1998; Simon et al., 1992; Sin et al., 2002). However, manipulation of NMDAR activity in mature mammals does not cause large structural alterations (Kalb, 1994). We argue here that particular combinations of NMDAR subunits underlie these different age-dependent functions of the receptor.

NMDARs are composed of two obligatory NR1 subunits, plus two NR2A-D and/or NR3A-B subunits. The precise subunit combination determines the physiological and pharmacological properties of the receptor, their binding partners and downstream signaling effects (Sheng and Kim, 2002; Cull-Candy and Leskiewicz, 2004; van Zundert et al., 2004b). Important details about the function of the NR2C-D (Brickley et al., 2003; Harney et al., 2008; Iijima et al., 2008) and NR3A-B subunits (Nishi et al., 2001; Ulbrich and Isacoff, 2008; Roberts et al., 2009) are only recently emerging, while considerable evidence points to a central role of the NR2A and NR2B subunits in regulating plasticity. Because a close correlation was found between the timing of the critical period for structural plasticity and the switch from slow NR2B-mediated currents to fast decaying NR2A-mediated currents across a variety of brain regions (Carmignoto and Vicini, 1992; Flint et al., 1997; Roberts and Ramoa, 1999), it has been proposed that the NR2B subunit allows dynamic synaptic changes, whereas the NR2A subunit increases temporal resolution hereby inducing refinement of the CNS (Scheetz and Constantine-Paton, 1994). A requirement for NR2B in dendritic patterning in vivo was indeed shown recently with the use of mosaic analysis with double markers (MADM) to knock-out NR2B in isolated single neurons (Espinosa et al., 2009). To gain insights in the specific roles of NR2A- versus NR2B-containing receptors in dendrite development in single Xenopus tectal neurons, Ewald et al. (2008) used overexpression and morpholine-mediated knock-down of NR2A and NR2B subunits. Although they
showed that dendrite development was affected by manipulation of both NR2A and NR2B, the results are rather complex. One important reason for this may relate to the presence of mixed NMDAR complexes: NR1, NR2A and NR2B subunits exist in vivo at synaptic sites not only as di-heteromeric (e.g. NR1/NR2A or NR1/NR2B), but also as tri-heteromeric receptors (e.g. NR1/NR2A/NR2B) that are abundantly expressed during development (Sheng et al., 1994; Sans et al., 2000; Dunah and Standaert, 2003; van Zundert et al., 2004b; Al-Hallaq et al., 2007). The presence of mixed NMDAR complexes is also likely to play an important role in the wide debate whether NR2A and NR2B subunits have differential roles in LTP and LTD (MacDonald et al., 2006; Yashiro and Philpot, 2008).

VSCNs offer a unique opportunity to bypass the presence of tri-heteromeric NMDAR complexes, because the expression of both NR2A and NR2B is down-regulated in culture, resulting in NR2A and NR2B “null” neurons (Mi et al., 2004). To test the hypothesis that replacement of NR1NR2B-receptors by NR1NR2A-receptors during development may contribute to the down-regulation of structural changes as the CNS mature, we introduced exogenous NR2A or NR2B in VSCNs, together with DsRed to label the neuronal dendritic arbors. Our results show for the first time directly that NR1NR2B-receptors, but not NR1NR2A-receptors, induce dendritic branching in VSCNs. Analysis of well characterized mutants of RasGRF1 (Krapivinsky et al., 2003) further suggests that NMDAR actions on dendritic branching depends on the interaction between NR2 subunits and this signaling protein. Importantly, our results were also corroborated in hippocampal neurons.
Experimental Methods

Neuronal cultures and transfections

All protocols involving rodents were in accordance with the NIH guidelines and were approved by the Ethical and Bio-security Committees of the University of Concepción. Pregnant Sprague-Dawley rats were deeply anesthetized with CO₂ and cultures of VSCNs and hippocampal neurons were prepared from E14 and E20 pups, respectively (Mi et al., 2004; van Zundert et al., 2004a). Briefly, hippocampi and whole spinal cords were excised and placed into ice-cold HBSS (Gibco 14185-052) containing 50 μg/ml penicillin/streptomycin (Gibco 15070-063). The dorsal part of the spinal cord was removed using a small razor blade. The hippocampus and the ventral spinal cord were minced and incubated with pre-warmed HBSS containing 0.25% trypsin (Gibco 15090-046) in an incubator for 20 min at 37°C. After enzymatic treatment, the cells were transferred to a 15 ml tube containing neuronal growth media (70% MEM (Gibco 11090-073), 25% Neurobasal media (Gibco 21103-049), 1% N2 supplement (Gibco 17502-048), 1% L-glutamine (Gibco 25030-081), 1% penicillin-streptomycin (Gibco 15070-063), 2% horse serum (Hyclone SH30074.03; lot AQH24495) and 1 mM pyruvate (Sigma). After the cells precipitated, they were transferred to a new 15 ml tube containing 2 ml growth media and cells were resuspended by mechanical agitation through decreasing diameter fire-polished glass Pasteur pipette. After cell counting, 1x10⁶ cells were plated on freshly prepared poly-L-lysine-coated 6-well plates (1 mg/ml; 30.000-70.000 MW; Sigma P2636) in growth media and cultured for 3 days at 37°C under an atmosphere containing 5% CO₂. All VSCNs cultures were supplemented with 45 μg/ml E18 chick leg extract (Henderson et al., 1993). Fresh media was replaced every 3 days. Cultured VSCNs predominantly contained interneurons (SI Fig. 1), and motoneurons were excluded from analysis by discarding cells with somata larger than 30 μm and expressing more than 6 primary dendrites.

At 4 DIV, VSCNs were transfected using a novel CaPO₄ transfection protocol to reduce cell toxicity (Jiang and Chen, 2006). Briefly, after 1 hour of incubation at 5% CO₂, the cells were incubated for 20
min with transfection medium pre-equilibrated in a 10% CO₂ incubator to dissolve the DNA-CaPO₄ precipitates and then returned to a 5% CO₂ incubator till 11 DIV. The 7-day incubation allowed efficient transcription of the different constructs. Also neurons were healthy during this period, and growth of branches was robust. The constructs used to transfect VSCNs neurons included: plasmids coding for DsRed2 and GFP to visualize neuronal morphology in detail; plasmids coding for NR2A and NR2B (Luo et al., 2002); plasmids coding for NR2B-BD (aa 886-1310), a GFP protein fused to the minimal binding-domain (BD) of NR2B that interacts with RasGRF1 (Krapivinsky et al., 2003); and plasmids coding for RasGRF1-BD (aa 714-913), a GFP protein fused to the minimal binding-domain of RasGRF1 that interacts with NR2B (Krapivinsky et al., 2003). For some experiments, a Herpes Simplex Virus (HSV) amplicon vector p1005 was constructed, containing eGFP driven by a CMV promoter and containing NR2A or NR2B driven by IE 4/5.

In another set of experiments, 7 DIV hippocampal neurons were transfected with pSUPER vectors coding for NR2A-RNAi and NR2B-RNAi (Kim et al., 2005), and their morphology analyzed at 15 DIV. Since 15 DIV hippocampal neurons express both the NR2A and NR2B subunits, possible forming tri-heteromeric synaptic NMDAR complexes at this time (Fig. 4; SI Fig. 2A-C; Tovar and Westbrook, 1999), the RNAi’s allow to shift the expression towards either NR2A- or NR2B-rich receptor complexes. To corroborate the importance of the NR2B-RasGRF1 interaction found in VSCNs, 4 DIV hippocampal neurons were transfected with plasmids coding for NR2B-BD and RasGRF1-BD (Krapivinsky et al., 2003), and their morphology analyzed at 7 DIV. We specifically analyzed 7 DIV cultures, because at this time of development the hippocampal neurons express predominantly synaptic NR1NR2B receptors (Fig. 8; SI Fig. 2D-F; Tovar and Westbrook, 1999).
Electrophysiology

Whole cell patch-clamp recordings on VSCNs and hippocampal neurons were performed and analyzed as previously described (van Zundert et al., 2004a). Briefly, the external solution contained: 150 mM NaCl, 5.4 mM KCl, 2.0 mM CaCl$_2$, 2.0 mM MgCl$_2$, 10 mM HEPES (pH 7.4), and 10 mM glucose. Patch electrodes (4.5-6.5 MΩ) were filled with: 120 mM CsCl, 10 mM BAPTA, 10 mM HEPES (pH 7.4), 4 mM MgCl$_2$, and 2 mM ATP-Na$_2$. After formation of a high resistance seal and break-in (>1GΩ), whole-cell voltage signals were recorded with an Axopatch 200B amplifier (Molecular Devices). Pipette and whole-cell capacitance, and series resistance were compensated using amplifier circuitry. Signals were low pass-filtered (5 kHz) and digitized (5-40 kHz) on a PC using PClamp 9.2 software. Cells were held at -60 mV.

To record both synaptic and extrasynaptic NMDARs the external solution contained NMDA (1-1000 μM) and glycine (10-50 μM) but lacked Mg$^{2+}$. AMPA/KARs, GlyRs and GABA$_A$Rs were evoked by 100 μM glutamate, 100 μM glycine and 10 μM GABA, respectively. To apply increasing concentrations of the drugs rapidly (time constant, 100 ms), we used an array of external tubes (internal diameter, 200 μm) placed within 50 μm of the neuron. The solutions containing the ligands flowed continuously by gravity from the tubes, which were connected to a 20-ml reservoir. Drug application was started and terminated by horizontally moving, with the aid of a micromanipulator.

To analyze synaptic neurotransmitter receptors, we recorded synaptic currents mediated by NMDARs, AMPAR or GABA$_A$R/GlyRs (IPSCs), and events were isolated pharmacologically using a mixture of antagonists against AMPAR (2 μM CNQX), NMDARs (20 μM D-APV and 2 mM Mg$^{2+}$), GlyRs (750 nM strychnine) or GABA$_A$Rs (2 μM bicuculline), as previously described for cultured spinal cord neurons (van Zundert et al., 2004a; Carrasco et al., 2007). Thus, spontaneous NMDAR-mediated EPSCs were isolated in Mg$^{2+}$-free ACSF by the addition of CNQX, strychnine...
and bicuculline. The ACSF also contained the NMDAR co-activator glycine (10 µM). We confirmed that the remaining synaptic currents were mediated only by NMDARs, as they were completely abolished by addition of D-APV to the ACSF. Spontaneous AMPAR-mediated EPSCs were isolated by the addition of D-APV, strychnine and bicuculline. They were confirmed as AMPAR-mediated currents by a complete blockade with CNQX. Spontaneous IPSCs were isolated by the addition of D-APV and CNQX, and confirmed as GABA<sub>A</sub>R/GlyR-mediated currents by a complete blockade with strychnine and bicuculline. For recordings of miniature AMPAR- and IPSC-mediated currents, the sodium channel blocker TTX (500 nM) was also added to the bath.

Every identified synaptic event collected during the test intervals displaying an amplitude above the background noise was analyzed using MiniAnalysis 6.0 software (Synaptosoft). Synaptic currents were characterized by the following parameters: peak amplitude, rise time (from 10%–90% of peak amplitude) and decay-time. Decay-time was determined by fitting the scaled averaged synaptic current decay from 90%–10% peak amplitude with a single exponential and is given in ms throughout the text as decay interval measured at 0.37 peak amplitude of the fitted exponential.

**Morphological analysis**

Fluorescent labeled neurons were fixed in 4% paraformaldehyde and visualized with a confocal (Nikon C1) or a spinning disk (Olympus IX81) microscope. Stacks of 0.50 µm optical sections were acquired throughout whole neurons. Dendrite number and assessment of the length of each dendrite was performed as described previously using a semi-automated procedure in Metamorph software (Universal Imaging) (Charych et al., 2006). Images were contrast-enhanced to assure that all branches were analyzed, except primary branches with a length of ≤ 20 µm: To measure the length of each individual primary dendrite, every segment that arose at the soma boundary was digitally marked until its terminus. If the primary dendrite did branch, the largest segment after the branching was selected to be part of the
primary branch, while the shorter branch would be analyzed as a secondary branch. The same methodology was performed to distinguish between secondary and tertiary branches. To reduce the possibility of researcher bias, analyses were performed by an investigator blinded to the experimental conditions. Sholl analysis was performed with ImageJ using a special plug-in designed by the laboratory of Dr. Anirvan Ghosh, UCSD, San Diego (available at http://www-biology.ucsd.edu/labs/ghosh/software/index.html).

Western blot

To determine the specificity and efficiency of RNAi constructs, HEK293 cells were transfected with either NR2A or NR2B and NR2A-RNAi or NR2B-RNAi, as indicated. Two days later, whole cell lysates were prepared with a buffer containing 50 mM Tris HCL, 100 mM NaCl, 2 mM EDTA, 0.1% SDS, proteinase and phosphatase inhibitors. Samples (20 μg) were run on a 10% SDS-PAGE gel, and transferred to a nitrocellulose membrane. The membrane was blocked with 5% milk and incubated overnight at 4°C with primary antibodies against NR2A (1:500, polyclonal, Covance, cat. PRB-513P), NR2B (1:500, polyclonal, Covance, PRB-512P), and tubulin (1:2000, Sigma, USA, cat. T5168) as loading control. After washing in PBS, membranes were incubated with an HRP-conjugated secondary antibody (1:5000; Santa Cruz Biotechnology) for 1 hour at RT, washed and incubated with ECL solution (Perkin Elmer) for 1 min, and exposed for 1-3 min on Biorad films.

Statistical Analyses

An ANOVA followed by post hoc Tukey tests were used to detect significant changes. Student's t tests were used when comparing the response of two populations to individual treatments. In all figures, error bars represent the SEM; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.
Results

VSCNs lack functional NMDARs but express AMPARs, GABA\(_A\)Rs and GlyRs

Previous studies using immunocytochemical and Western blot analyses showed that the expression of both NR2A and NR2B (but not NR1) is down-regulated in cultures of VSCNs (Mi et al., 2004). Here we performed whole-cell patch-clamp recordings and show that in control VSCNs (11 DIV) no apparent NMDAR-mediated current was detected even when 1000 \(\mu\)M NMDA, a saturating concentration of NMDA, was applied exogenously together with glycine (10 \(\mu\)M) and in the absence of Mg\(^{2+}\) (Fig. 1A left traces; Fig. 1C left traces). Raising glycine concentrations up to 50 \(\mu\)M produced similar results (not shown). In addition, recordings of synaptic activity in control VSCNs (n=15) did not exhibit any spontaneous NMDAR EPSCs (sNMDA) (Fig. 1B upper traces). In contrast, electrophysiological recordings show that cultured VSCNs abundantly express AMPA, glycine and GABA\(_A\) receptors (Fig. 1A-B).

Functional NMDARs are formed at the post-synaptic membrane of VSCNs after expression of NR2A or NR2B

To allow the formation of either NR1NR2A- or NR1NR2B-receptors, VSCNs were transfected at 4 DIV with DsRed2 and either a plasmid encoding the NR2A or the NR2B subunit. At 11 DIV, formation of functional NMDARs was analyzed by electrophysiology combined with pharmacological assays. First, we measured the whole-cell current peak amplitude in control neurons and those expressing the NR2A and NR2B subunit after exogenous application of increasing concentrations of the ligand NMDA (1-1000 \(\mu\)M) together with the co-agonist glycine (10 \(\mu\)M) and in the absence of Mg\(^{2+}\). Dose-response curves (Fig. 1C) from NR2A (middle traces) and NR2B (right traces) expressing neurons resulted in gradually enhanced NMDA currents with EC\(_{50}\)'s of 83±11 \(\mu\)M (n=4) and 53±4 \(\mu\)M (n=4), respectively.
In contrast, control neurons showed no significant current values upon NMDA application: The left traces in Figure 1C are obtained from the control neuron exhibiting the largest NMDA-mediated currents detected. The maximal current amplitude density activated with 1000 μM NMDA was 0.5±0.2 pA/pF in control neurons (n=4), 26±2 pA/pF in NR2A expressing neurons (p<0.001 versus control, n=4), and 27±4 pA/pF in NR2B expressing neurons (p<0.001 versus control; n=4) (Fig. 1D). Co-administration of NMDA with 50 μM glycine yielded similar results with maximal current amplitude densities of 0.9±0.2 pA/pF in control neurons (n=4), 24±3 pA/pF in NR2A expressing neurons (p<0.001 versus control, n=4), and 29±1 pA/pF in NR2B expressing neurons (p<0.001 versus control; n=4).

Next, we recorded spontaneous NMDAR mediated postsynaptic currents (sNMDA). As expected, neurons expressing the NR2A subunit presented events with fast decay-time kinetics (Fig. 2A, upper traces) that were largely insensitive to the NR2B antagonist ifenprodil (middle traces), while VSCNs expressing the NR2B subunit displayed events with slow decay-time kinetics (Fig. 2B, upper traces) that were selectively abolished by ifenprodil (middle traces). All currents were blocked by the selective NMDAR antagonist D-APV (Fig. 2A-B, lower traces). Figure 2C shows amplitude histograms from 7-8 VSCNs expressing either the NR2A (white bars) or the NR2B subunit (black bars). Summary graphs (Fig. 2D) including the averaged sNMDA current amplitude (left graph), frequency (middle graph) and decay-time (right graphs) are also shown. NR2B–expressing neurons display NMDAR-mediated currents with significantly larger amplitudes (p<0.05) and slower decay kinetics (p<0.001) compared to NR2A-expressing neurons. The frequency and rise-time (not shown) were similar between NR2A and NR2B-expressing neurons. Together, these results demonstrate that control VSCNs lack NMDARs but that functional NR1NR2A- and NR1NR2B-receptors are formed at the postsynaptic membrane of VSCNs after expression of NR2A or NR2B, respectively. Same conclusions were reached independently of the approach used to express NR2A and NR2B in VSCNs, as similar results were obtained when neurons were infected with herpes simplex virus.
(HSV) carrying the GFP gene together with either the NR2A (HSV-GFP-NR2A) or the NR2B subunit gene (HSV-GFP-NR2B) (data not shown).

Expression of NR2B, but not NR2A, promotes dendritic branching of VSCNs

To directly assess whether the NR2A and NR2B subunits have different roles in regulating dendritic branching, we counted the primary, secondary and tertiary branches of 11 DIV VSCNs transfected with DsRed2 alone (called “control”; Fig. 3A, left image) or DsRed2 together with either NR2A (center image) or NR2B (right image). As it can be observed in the images and graphs of figure 3, those neurons expressing NR2B (Fig. 3B, black bars) displayed a more complex dendritic architecture relative to control cells (white bars), with significant increases in the number of secondary (p<0.05 NR2B versus control; p<0.001 NR2B versus NR2A) and tertiary branches (p<0.001 NR2B versus control and NR2A). Similar results were found when neurons were infected with HSV-GFP-NR2B (data not shown). In contrast, VSCNs transfected with NR2A (Fig. 3B, grey bars) or infected with HSV-GFP-NR2A (not shown) displayed a dendritic architecture comparable to that of control cells, with no significant changes in the number of primary, secondary or tertiary branches (p>0.05 NR2A versus control). Because in all our experiments using VSCNs the number of primary branches remained always unaltered, these measurements will no longer be presented.

To further address the nature of the morphological changes we analyzed branch lengths. Averaged lengths of the primary, secondary and tertiary branches were not significantly different between control, NR2A- and NR2B-expressing VSCNs (p>0.05; not shown). In contrast, summation of the lengths of all dendritic branches indicated that NR2B-expressing neurons had an approximately 2 fold increase in total outgrowth (p<0.001 NR2B versus control and NR2A; Fig. 3C). To analyze the distribution of branches across the entire dendritic tree, we performed Sholl analysis by measuring the number of branches that cross a set of concentric circles drawn around the cell soma at increasing radial distances.
(inset, Fig. 3D; 10 µm Sholl bins). Whereas NR2A-expressing neurons did not induce changes in Sholl analysis (Fig. 3D), neurons expressing NR2B increased branch density independent of the distance from the soma compared to control (Fig. 3E). These results indicate that NR1NR2B-receptor expression is associated with a robust increase in the dendritic branching in VSCNs, without changing branch elongation. By contrast, NR1NR2A-receptors have little effect on the dendritic architecture.

**Knockdown of NR2B and NR2A alters dendritic branch development in hippocampal neurons**

Given the observation that NR2B expression in VSCNs increases branching, we hypothesized that reduction of NR2B subunit expression in neurons that express tri-heteromeric receptor complexes would decrease dendritic branch formation, whereas reduction of NR2A subunit expression would increase branching. To test this hypothesis, we used 15 DIV hippocampal neurons because they express both the NR2A and NR2B subunits, possibly forming tri-heteromeric synaptic NMDAR complexes (Tovar and Westbrook, 1999). In agreement, our electrophysiological recordings showed that 15 DIV hippocampal neurons present NMDAR-mediated sEPSCs with intermediate decay-time kinetics (Fig. 4A, upper trace) that were partly sensitive to the NR2B antagonist ifenprodil (middle traces) and completely blocked by D-APV (lower traces). Additional recordings of evoked NMDAR currents (eNMDA; SI Fig. 2A-B) and Western blot analysis (SI Fig. 2C) also corroborate the presence of both NR2A and NR2B subunits in 15 DIV hippocampal neurons.

To knock-down the expression of NR2A and NR2B, we used previously characterized plasmid-based RNA interference (RNAi) against NR2A and NR2B (Kim et al., 2005). Western blot analysis (SI Fig. 3A) and electrophysiology (SI Fig. 3B-C) of HEK293 cells confirmed the effectiveness of both RNAi’s. To shift the expression towards either NR2A- or NR2B-rich receptor complexes, hippocampal neurons were transfected with DsRed2 alone (control), or together with NR2B-RNAi, or with NR2A-RNAi at 7 DIV and analyzed at 15 DIV. In support of our proposed role of the NR2B...
subunit in branch formation, we found that hippocampal neurons transfected with NR2B-RNAi displayed a small but significant decrease in the number of secondary branches (p<0.001; Fig. 4B, middle image; Fig. 4C, light grey bars) relative to control neurons (Fig. 4B, left image; Fig. 4C, white bars). Sholl analyses showed that the branch reduction associated with NR2B-RNAi expression was independent of the distance from the soma (Fig. 4F). NR2B-RNAi expression had no effect on the number of primary or tertiary branches of hippocampal neurons (p>0.05; Fig. 4C), or on the total branch length of the dendritic tree (Fig. 4E).

Since NR2B-RNAi also affects endogenous NR2A expression (Kim et al., 2005), the reduced number of secondary branches observed in Figure 4C could be caused by a reduction in the expression of the NR2A subunit. This seems unlikely, however, because when neurons were co-transfected with both NR2B-RNAi and NR2A (Fig. 4C, dark grey bars), we detected no significant change in branch number relative to levels obtained with NR2B-RNAi alone (p>0.05; Fig. 4C, light grey bars).

To evaluate the role of NR2A in regulating branch formation in hippocampal neurons, we used NR2A-RNAi (Fig. 4B, right image). Compared to control (Fig. 4D, white bars), expression of NR2A-RNAi (Fig. 4D, light grey bars) markedly increased the number of secondary (p<0.001) and tertiary (p<0.001) branches. Co-expression of NR2A-RNAi with NR2B (Fig. 4D, dark grey bars) did not significantly increase branching relative to levels obtained with NR2A-RNAi alone (light grey bars; p>0.05). In agreement with the significant enhancement in the number of secondary and tertiary branches, NR2A-RNAi also caused an approximately 2.5 fold increase in total branch outgrowth (p<0.001; Fig. 4E). Sholl analyses indicated that branch addition was present throughout the dendritic arbor (Fig. 4F). Together, these hippocampal RNAi experiments support our observations in VSCNs that the NR2A and NR2B subunits have differential roles in regulating dendritic branch formation.
Expression of NR2A and NR2B in VSCN increases mAMPA and mIPSC

Our results showing that NR1NR2B-receptor expression is associated with a robust increase in the dendritic branching in VSCNs (Fig. 3) is interesting, however, it is important to determine whether the newly formed branches in NR2B-expressing VSCNs establish functional excitatory synapses. To demonstrate this, we recorded miniature synaptic currents mediated by AMPARs (mAMPA) in control and transfected VSCNs (Fig. 5A). Figure 5B shows amplitude histograms from 5-6 control VSCNs (white bars) and from those expressing either the NR2A (grey bars) or the NR2B subunit (black bars). Summary graphs (Fig. 5C) of averaged mAMPA current amplitude (left graph) and frequency (right graph) are also shown. We found that mAMPA frequency increased in NR2B-expressing VSCNs (Fig. 5C, right graph, black bars) relative to control neurons (p<0.001; Fig. 5C, white bars). The lack of changes in the amplitude of mAMPA in the NR2B-expressing neurons (p>0.05; Fig. 5C, left graph) indicates that the number of AMPARs per synapse was not altered. On the contrary, NR2A-expressing VSCNs (grey bars) displayed an increase in mAMPA amplitude (p<0.001; Fig. 5C, left graph) and frequency (p<0.05; Fig. 5C, right graph), indicating that NR2A promotes the incorporation of AMPARs at the synapse as established by previous studies (Kim et al., 2005; Hall et al., 2007). We also recorded miniature inhibitory synaptic activity mediated by GlyRs/GABA<sub>A</sub>R (mIPSC) and found that the frequency (Fig. 5D, right graph), but not the amplitude (Fig. 5D, left graph), of mIPSCs was significantly increased in both NR2A and NR2B-expressing VSCNs relative to control.

NR2B-induced structural changes are prevented by NMDAR and AMPAR antagonists

To investigate whether the NR2B-induced structural changes in VSCNs require functional NMDARs or AMPARs, VSCN cultures were chronically treated with D-APV (10 μM) or CNQX (2 μM) from the day of transfection. These antagonists had no effect on the number of primary (not shown),
secondary (upper bar histograms) or tertiary (lower bar histograms) branches when applied to
cultures of control VSCNs (Fig. 6A) or those transfected with NR2A (Fig. 6B). By contrast, when D-
APV or CNQX was applied to NR2B expressing VSCNs, marked reduction in the number of
secondary (p<0.001, Fig. 6C, upper bar histograms) and tertiary branches (p<0.001, Fig. 6C, lower
bar histograms) was observed. Chronic treatment with the open-channel blocker MK-801 also
significantly inhibited NR2B-induced branch formation (data not shown). These results indicate that
activity mediated by both NMDARs and AMPARs is required for NR2B-induced branching.

We also used TTX (1 μM) to assess whether chronic blockage of voltage-gated Na⁺ channels
(Naᵥ) could affect branching in VSCNs. TTX was unable to prevent NR2B-induced dendritic branch
formation (Fig. 6C). TTX had also no effect on the morphology of control (p>0.05; Fig. 6A) or
NR2A-expressing neurons (p>0.05; Fig. 6B). These results indicate that the morphological changes
induced by NR1NR2B receptors are independent of the action potential-mediated activity of neurons
in culture. Studies in developing retinal ganglion neurons have also shown that dendritic remodeling
is independent of Naᵥ activity (Wong et al., 2000; Kerschensteiner et al., 2009).

Disruption of NR2B-RasGRF1 interaction inhibits dendritic branching

As shown in Figure 3, we found that NR2B expression resulted in a more complex dendritic
architecture compared to NR2A-expressing neurons. How do NR1NR2A-receptors and NR1NR2B-
receptors differentially regulate branch formation? One possible mechanism is that NR2B-containing
NMDARs facilitate a larger calcium influx at the postsynaptic membrane compared to NR2A-
containing receptors (Sobczyk et al., 2005), due to their different biophysical properties (Carmignoto
and Vicini, 1992; Tang et al., 1999). Strikingly, however, NR2A-expressing VSCNs did not display
more dendritic branches compared to control neurons (Fig. 3B) that lack NMDAR currents (Fig. 1).
These results prompted us to examine whether a particular downstream signaling pathway that is
coupled to NR1NR2B-containing receptors is required to induce dendritic branch development. Given that RasGRF1 is a key signaling component in the regulation of several forms of neuronal plasticity, specifically associates with the NR2B subunit (Krapivinsky et al., 2003; Li et al., 2006), and controls neurite extension in PC12 cells (Yang and Mattingly, 2006), we hypothesized that the binding of RasGRF1 to NMDARs is necessary for inducing branch formation. To impede the interaction between the NR2B subunit and RasGRF1, VSCNs were transfected with DsRed2 and NR2B (Fig. 7A, left image), together with a plasmid coding for the minimal binding-domain (BD) of RasGRF1 that interacts with NR2B (RasGRF1-BD, middle image), or with a plasmid coding for the minimal binding-domain of NR2B that interacts with RasGRF1 (NR2B-BD, right image). Both constructs have been previously characterized in detail (Krapivinsky et al., 2003). As can be observed in the images and in the graphs of Figure 7, compared to neurons that express only NR2B (white bars), co-expression with either RasGRF1-BD (p<0.001; light grey bars) or with NR2B-BD (p<0.001; dark grey bars) prevented the increase in the number of secondary (Fig. 7B) and tertiary (Fig. 7C) branches.

The above results suggest that a functional association of RasGRF1 with the NR2B subunit is important during dendritic branch formation. However, it is possible that NR2B-BD and RasGRF1-BD proteins retain binding sites for additional proteins and may sequester them, limiting hereby their possible interaction with the NMDAR. In particular, the C-terminus of the NR2B subunit, and thus also the C-terminus of NR2B-BD, contains a binding site for CaMKII (Barria and Malinow, 2005). To maintain the ability of NR2B-BD to sequester RasGRF1, but yet specifically reduce its interaction with CaMKII, we generated the mutant isoform NR2B-BD-RS/QD by substituting respectively Arg1300 and Ser1303 with Gln and Asp in the NR2B-BD (SI Fig. 4A). This same mutation was previously used to reduce specifically the interaction between NR2B and CaMKII (Barria and Malinow, 2005). Compared to neurons that expressed NR2B plus NR2B-BD, co-expression of NR2B
with NR2B-BD-RS/QD displayed a similar number of dendritic branches (SI Fig. 4), indicating that NR2B-BD limits branch formation independent of the NMDAR-CaMKII association.

Finally, we also assessed whether the NR2B-RasGRF1 interaction is required for dendritic branch development in hippocampal neurons. For these studies we specifically analyzed 7 DIV hippocampal neurons, because in agreement with previous reports (Tovar and Westbrook, 1999), our electrophysiological recordings of NMDAR-mediated sEPSCs indicate that NR2B-containing NMDARs are predominantly expressed at this time. Thus, we found that sNMDA display slow decay-time kinetics (Fig. 8A, upper trace) that are completely blocked by ifenprodil (middle traces) and D-APV (lower traces). Additional recordings of eNMDA (SI Fig. 2D-E) and Western blot analysis (SI Fig. 2F) also corroborate the presence of mainly the NR2B subunit in 7 DIV hippocampal neurons. To block the NR2B-RasGRF1 interaction, 4 DIV hippocampal neurons were transfected with DsRed2 alone (Fig. 8A, left image), plus NR2B-BD (middle image), or plus RasGRF1-BD (right image) and their morphology analyzed at 7 DIV. As it can be observed in the images and in the graphs of Figure 8, expression of NR2B-BD (Fig. 8C, grey bars) significantly decreased the number of secondary (p<0.001) and tertiary branches (p<0.001) compared to control hippocampal neurons (white bars). Similarly, expression of RasGRF1-BD (Fig. 8D, dark grey bars) blocked the development of secondary (p<0.001) and tertiary branches (p<0.001) compared to control (white bars). Together, these results indicate that the interaction between NR2B and RasGRF1 is required for normal dendritic branch formation in both VSCNs and hippocampal neurons.
Discussion

The importance of NMDARs in developmental biology, synaptogenesis, and neuronal plasticity has been well established. However, the mechanisms that link specific NMDAR subunits with these processes remain largely unknown, mainly because NMDARs can be present as di-heteromeric (e.g. NR1/NR2C) and tri-heteromeric complexes (e.g. NR1/NR2A/NR2B). Because the expression of NMDAR subunits, including NR2A and NR2B, is completely down-regulated in VSCNs (Mi et al., 2004) and functional NMDARs are lacking (our study), these naturally ‘NMDAR null’ neurons offer a powerful model system for dissecting out the contribution of individual subunits in neuronal plasticity and dendrite development.

VSCNs as a model system to study differential roles of NR1NR2A versus NR1NR2B receptors.

Functional and structural plasticity in the CNS is apparent in early life, but is markedly diminished with maturation. Considerable evidence points to a role for the NMDAR in the remodeling of the neuronal architecture during early development (Kalb, 1994; Inglis et al., 1998; Simon et al., 1992; Sin et al., 2002). However, the molecular basis for decreased structural remodeling in mature neurons is not well understood. The strong correlation between the developmental switch from the NR2B subunit to NR2A led investigators to hypothesize that NR1NR2B-receptors allow structural reorganization of connections, whereas NR1NR2A-receptors promote map stability. However, it has been difficult to address this hypothesis. Ectopic expression and knock-down of the endogenous NR2A or NR2B subunits led to complex results (Ewald et al., 2008), possibly because overexpression or knock-down experiments only shift the expression towards NR2A- or NR2B-rich receptor complexes, without eliminating the mixed NR1/NR2A/NR2B receptor complexes that are expressed abundantly during development (Sheng et al., 1994; Sans et al., 2000; Dunah and Standaert, 2003; Al-Hallaq et al., 2007).

The use of pharmacology also presents a challenge as the effectiveness of the NR2B-specific antagonist
ifenprodil is significantly reduced when this subunit is part of the mixed NR1/NR2A/NR2B receptor complex (Williams, 2001). Moreover, the NR2A antagonist NVP-AAM077 lacks specificity and reportedly affects approximately 40% of the NR2B-mediated currents in NR2A knock-out mice (De Marchena et al., 2008).

To investigate the specific role of the NR2A versus NR2B subunit in regulating dendritic branch formation, we introduced NR2A or NR2B subunits in ‘NMDAR null’ VSCNs. Using this system we provide evidence that NR1NR2B-receptors induce dendritic branching in VSCNs. This was corroborated in hippocampal cultures by knocking-down the NR2B subunit. In contrast, the role of the NR2A subunit in branch formation remains to be fully established: knock-down of NR2A increased branch formation in hippocampal neurons whereas NR2A expression in VSCNs did not significantly reduce branching. It is possible that the NR2A subunit does not directly inhibit dendritic growth, but may indirectly regulate the synaptic location of the NR2B subunit. Thus, in hippocampal neurons that contain abundant tri-heteromeric NR1/NR2A/NR2B receptors, knockdown of NR2A would effectively increase the number of NR1NR2B di-heteromers at the synapse, thereby inducing branch formation.

Our results suggest that, as the nervous system matures, replacement of NR1NR2B-receptors by NR1NR2A-receptors may contribute to the down-regulation of structural changes. Other regulatory subunits such as NR2C-D and NR3A-B may also participate in this process. The NR2C, NR2D and NR3B subunits are expressed in spinal cord cultures (Mi et al., 2004; Prithviraj and Inglis, 2008), but it seems unlikely that functional di-heteromeric NR1NR2C/D or NR1NR3A/B glutamate-sensitive NMDARs are effectively formed, since we did not observe spontaneous NMDAR mediated activity nor significant NMDAR-evoked currents in control VSCNs (Fig. 1). However, we can not rule out the possibility that tri-heteromeric receptors are assembled in VSCNs when NR2A or NR2B subunits are introduced exogenously. In particular, Nishi et al., (2001) suggested that NR1/NR2A-B/NR3B receptor complexes limit dendritic branching during maturation since (1) NR3B acts as a putative dominant-
negative subunit that reduces NMDAR activity, and (2) its expression in up-regulated in motoneurons during development. Unexpectedly, however, recent studies showed that overexpression of NR3B actually increased branch formation in cultured motoneurons (Prithviraj and Inglis, 2008), leaving unresolved the issue of whether the branching in NR2B-expressing VSCNs is due, at least in part, to the incorporation of the endogenous NR3B subunit in NR1NR2B-receptors. Nevertheless, as in NR2B-expressing VSCNs, ectopic expression of the NR2A subunit likely interacts with NR3B subunits and the finding that expression of NR2A did not display increased branching compared to control, does not support the idea that NR3B-containing triheteromeric NMDARs are formed in our VSCNs. Single channel recordings may define whether NR1NR2ANR3B and NR1NR2BNR3B receptor complexes are formed in these VSCNs, however, those studies are beyond the scope of the present work.

Electrophysiological recordings indicate that, as with the NR3 subunits, the NR2C and NR2D subunits can co-assemble with NR1/NR2A-B to form tri-heteromeric receptors with altered functional properties (Cull-Candy and Leskiewicz, 2004). How these subunits can regulate plasticity is not clear: The NR2C expression is confined almost entirely to the cerebellum, thalamus and olfactory bulb (Wenzel et al., 1997), whereas NR2D-containing tri-heteromeric receptors are restricted to the extrasynaptic membrane (Brickley et al., 2003). Interestingly, recent studies indicate that extrasynaptic multimeric NR2D-containing NMDARs are recruited to the synapse site during LTP in hippocampal granule cells (Harney et al., 2008) and the presence of the NR2D subunit reduces Mg$^{2+}$ block of NMDARs in maturing motoneurons (Arvanian et al., 2004).

Possible mechanisms underlying NR2B-dependent branching

The opposite structural changes induced by NR2A and NR2B may be regulated by their differential association with other receptors (e.g. AMPARs), scaffolding proteins (e.g. PSD-95, SAP102, PSD-93) and/or signal transduction complexes (e.g. RasGRF1 and CaMKII) (Strack et al., 2000; Krapivinsky et
Our finding that the frequency of AMPAR-mediated transmission is increased by either NR2A or NR2B in VSCNs does not support a direct role for AMPARs in regulating NR2B-specific dendritic branch formation. However, we can not discard the possibility that VSCNs expressing NR2A or NR2B subunits regulate the membrane expression of different AMPAR subunits, such as GluR1 or GluR2, each coupled to distinct signaling pathways (Kim et al., 2005). Indeed, recent studies show that GluR1 and GluR2 differentially regulate dendritic complexity in cultured motoneurons (Prithviraj et al., 2008). However, similar experiments in cultured neocortical neurons did not show morphological differences induced by GluR1 and GluR2 (Chen et al., 2009).

Our data indicate that NR1NR2B-receptors promote branch formation, at least in part, via RasGRF1. RasGRF1 is a guanine nucleotide exchange factors with dual specificity for both Ras and Rac GTPases that is involved in the regulation of several forms of neuronal plasticity (Krapivinsky et al., 2003; Li et al., 2006). Studies in vitro and in vivo indicate that RasGRF1 couples NMDARs, specifically via the NR2B subunit, preferentially to Ras which in turn activates Erk and cAMP response element-binding protein (CREB) signaling (Tian et al., 2004; Krapivinsky et al., 2003; Kim et al., 2005; Ivanov et al., 2006). Our results that NR1NR2A-receptors do not promote dendritic branch development in VSCNs (Fig. 3) and hippocampal neurons (Fig. 4) may be explained by the lack of interaction between NR2A and RasGRF1 (Krapivinsky et al., 2003). In adult neurons dominated by NR2A expression, the limited interactions between synaptic NMDARs with RasGRF1 might therefore account for the decreased dendritic arbor development.

In conclusion, the dendritic branch formation observed in our experiments is best explained by interactions between NR2B and the signaling molecule RasGRF1. This interaction is reduced during development due to a down-regulation of NR2B subunits and increased expression of NR2A subunits.

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This switch in receptor subunits might underlie, at least in part, the restricted dendritic arbor development in the adult CNS.
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Figure Legends

Figure 1. VSCN cultures lack functional NMDARs, but express AMPARs, GlyRs and GABA\(_A\)Rs. Whole-cell patch-clamp recordings of 11 DIV VSCNs. Neurons were held at -60 mV. Mg\(^{2+}\) was eliminated from the external solution for the recordings of NMDARs. A, Representative traces of control neurons in the presence of exogenous NMDA, L-glutamate, glycine, or GABA are shown. B, Representative example traces of control VSCNs show recordings of the spontaneous activity mediated by NMDARs (sNMDA), AMPARs (sAMPA), GlyRs (sGlycine) or GABA\(_A\)Rs (sGABA). Note that no significant NMDAR-mediated currents (either evoked or spontaneous) are observed in VSCNs, whereas large currents mediated by glycine, GABA and glutamate are detected. C, Traces of control neurons (left), NR2A- (middle) and NR2B- (right) expressing VSCNs were obtained with the indicated concentrations of NMDA. D, Quantification of averaged current density (pA/pF) of control (n=4), NR2A- (n=4) and NR2B-expressing (n=4) neurons is shown. Figure shows Mean±SEM. ***p<0.001 (ANOVA).

Figure 2. sNMDA is generated in NR2A and NR2B transfected VSCNs. A-B, Whole-cell patch-clamp recordings (Vm=-60mV) are shown of pharmacologically isolated sNMDA in a Mg\(^{2+}\) free solution from 11 DIV VSCNs expressing since 4 DIV the NR2A (A) or the NR2B subunit (B). The averages of 40-50 sNMDA current events are also shown (right traces) with an indication of the decay-time of the single exponential curve used to fit the current decay-time. Expression of NR2A generates typical fast sNMDA, which are largely insensitive to ifenprodil (IFE; 3 µM), whereas NR2B expression produces typical slow sNMDA, which are inhibited by ifenprodil. Both currents are blocked by D-APV (20 µM). C, Summary frequency histograms of NR2A- (white bars, n=8) and NR2B-expressing neurons (black bars, n=7). D, Averaged peak amplitude (left), frequency (middle)
and decay-time (right) of VSCNs expressing NR2A or NR2B. Each symbol represents mean ± S.E.M. obtained from 7-8 neurons. *p<0.05, ***p<0.001 compared to NR2A (t-test).

**Figure 3. Expression of NR2B, but not NR2A, promotes dendritic branching in VSCNs.** A, Images of representative VSCNs expressing pDsRed2 alone (control; left) or with either NR2A (middle) or NR2B (right). Inset shows amplification of the boxed area. Note that NR2B-expressing neurons display a more complex dendritic architecture compared to either control or NR2A-expressing neurons. B, Quantification of averaged number of primary, secondary and tertiary branches of control (n=17), NR2A- (n=32) and NR2B-expressing (n=30) neurons is shown. C, Total outgrowth of control neurons and VSCNs expressing either NR2A or NR2B. Figures show Mean±SEM. *p<0.05, ***p<0.001 (ANOVA). D-E, Sholl analysis of control versus NR2A (D) and control versus NR2B (E) documents that cells expressing NR2B, but not NR2A, display an increased number of intersections, independent of the distance from the soma. Figures show Mean±SEM.

**Figure 4. Knockdown of NR2A and NR2B alters dendritic branching in hippocampal neurons.** A, Representative example traces of 15 DIV control hippocampal neurons (neurons held at -60 mV) show sNMDA with intermediate decay-time currents (upper traces) that are only partly sensitive to ifenprodil (3 μM IFE; middle trace). Currents are blocked by D-APV (lower traces). B, Images of representative hippocampal neurons expressing pDsRed2 alone (control; left), with either NR2B-RNAi (middle) or with NR2A-RNAi (right) are shown. Inset shows amplification of the boxed area. Note that neurons expressing NR2A-RNAi contain a more complex dendritic architecture compared to either control neurons, whereas NR2B-RNAi reduces branching. C, Quantification of averaged number of branches in control (n= 15) and in neurons expressing NR2B-RNAi alone (n= 17) or with NR2A (n=13) is shown. D, Quantification of averaged number of branches in control (n= 15) and neurons expressing NR2A-
RNAi alone (n=14) or with NR2B (n=17) is shown. E, Total outgrowth of control and RNAi treated neurons. Figures show Mean±SEM. ***p<0.001 (ANOVA). F, Sholl analysis of control and RNAi treated neurons. Cells expressing NR2A-RNAi, but not NR2B-RNAi, display an increased number of intersections, independent of the distance from the soma. Figure shows Mean±SEM.

Figure 5. Synaptic transmission is altered in VSCNs expressing NR2A and NR2B subunits. A, Recordings of miniature synaptic activity mediated by AMPARs (mAMPA) in VSCNs expressing pDsRed2 alone (control; upper traces), with either NR2A (middle traces), or with NR2B (lower traces). The averages of 40-50 mAMPA current events are also shown (right traces) with an indication of the decay-time of the single exponential curve used to fit the current decay-time. B, Summary frequency histograms of control (white bars, n=7), NR2A- (grey bars, n=7) and NR2B-expressing neurons (black bars, n=7). C-D, Quantifications of averaged amplitude (left) and frequency (right) of mAMPA (C) and mIPSC (D) are shown. For each treatment, 12-14 neurons were analyzed. Figures show Mean±SEM. *p<0.05, ***p<0.001 (ANOVA).

Figure 6. Dendritic branching is dependent on the activity of NMDARs and AMPARs. A-C, Quantifications of averaged number of secondary (upper) and tertiary (lower) branches of VSCNs expressing pDsRed2 alone (control; A), with either NR2A (B), or with NR2B (C). The cultures were chronically treated with D-APV (10 μM), CNQX (2 μM) or TTX (1 μM). For each treatment, 15-20 neurons were analyzed. Figures show Mean±SEM. ***p<0.001 (ANOVA).

Figure 7. Expression of NR2B-BD or RasGRF1-BD inhibits NR2B-dependent branching in VSCNs. A, Images of representative VSCNs neurons expressing DsRed2 with either NR2B alone (left), together with RasGRF1-BD (middle), or together with NR2B-BD (right). Inset shows amplification of
the boxed area. **B-C**, Quantifications of averaged number of secondary (B) and tertiary (C) branches of VSCNs expressing NR2B alone or together with the different binding-domain constructs are shown. Note that expression of NR2B-BD and RasGRF1-BD reduces NR2B-induced branching. At least 10 neurons were analyzed for each treatment. Figures show Mean±SEM. ***p<0.001 (ANOVA).

**Figure 8. Expression of NR2B-BD or RasGRF1-BD inhibits NR2B-dependent branching in hippocampal neurons.** **A,** Representative example traces of 7 DIV control hippocampal neurons (neurons held at -60 mV) show sNMDA with slow decay-time currents (upper traces) that are completely blocked by ifenprodil (IFE; middle trace) and D-APV (lower traces). **B,** Typical images of 7 DIV hippocampal neurons expressing DsRed2 alone (control, left), with either NR2B-BD (middle), or with RasGRF1-BD (right) are shown. Inset shows amplification of the boxed area. **C-D,** Quantifications of averaged number of primary, secondary and tertiary branches of VSCNs expressing DsRed2 alone or with NR2B-BD (C), or with RasGRF1-BD (D) are shown. Note that expression of NR2B-BD and RasGRF1-BD reduces branching. At least 12-14 neurons were analyzed for each treatment. Figures show Mean±SEM. ***p<0.001 (t-test).
Figure 1. Sepulveda et al.,

A

1000 μM NMDA 100 μM L-glutamate 100 μM glycine 10 μM GABA

B

sNMDA sAMPA sGlycine sGABA

C

Control NR2A NR2B

D

Current density (pA/pF)

***

0 5 10 15 20 25 30 35

400 pA 1 s

100 ms

***

Control NR2A NR2B
Figure 2. Sepulveda et al.,...
Figure 3. Sepulveda et al.
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Figure 5. Sepulveda et al.,
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