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The clinically available NMDA receptor antagonist, memantine, exhibits relative safety in the developing rat brain

Simon M. Manning^{a,b}, Griffin Boll^a, Erin Fitzgerald^a, Debra B. Selip^a, Joseph J. Volpe^a, and Frances E. Jensen^a

^aDepartment of Neurology, Children's Hospital Boston and Harvard Medical School, Boston, Massachusetts 02115, USA

^bDivision of Newborn Medicine, Brigham and Women's Hospital, Boston, Massachusetts 02115, USA

Abstract

The *N*-methyl-D-aspartate glutamate receptor (NMDAR) has been implicated in preterm brain injury (periventricular leukomalacia (PVL)) and represents a potential therapeutic target. However, the antagonist dizocilpine (MK-801) has been reported to increase constitutive neuronal apoptosis in the developing rat brain, limiting its clinical use in the developing brain. Memantine is another use-dependent NMDAR antagonist with shorter binding kinetics and has been demonstrated to be protective in a rat model of PVL, without effects on normal myelination or cortical growth. To further evaluate the safety of memantine in the developing brain, we demonstrate here that, in contrast to MK-801, memantine at neuroprotective doses does not increase neuronal constitutive apoptosis. In addition, there are no long-term alterations in the expression of NMDAR subunits, AMPAR subunits, and two markers of synaptogenesis, Synapsin-1 and PSD95. Evaluating clinically approved drugs in preclinical neonatal animal models of early brain development is an important prerequisite to considering them for clinical trial in preterm infants and early childhood.

Keywords

apoptosis; development; NMDA receptor; periventricular leukomalacia; synapses; memantine

1. Introduction

Memantine is an uncompetitive *N*-methyl-D-aspartate glutamate receptor (NMDAR) antagonist (Bormann, 1989), approved for clinical use in moderate to severe Alzheimer's disease (Forest Pharmaceuticals, 2003). It is similar to dizocilpine (MK-801) but with faster on-off kinetics (Chen et al., 1998; Lipton, 2007) as well as strong voltage and use-dependency (Parsons et al., 1995). Currently memantine is also being studied for efficacy in children with pervasive developmental disorder (PDD) (Owley et al., 2006; Chez et al., 2007). We have reported in a hypoxia-ischemia rat model of preterm brain injury (periventricular leukomalacia (PVL)) (Follett et al., 2000), that memantine attenuates both the myelination deficit and cortical loss (Manning et al., 2008), pathologies characteristic of human PVL (Volpe, 2009). However, many NMDAR antagonists including the anesthetic agents ketamine, and isoflurane, have been reported to cause a significant increase in

neuronal constitutive apoptosis in immature rodents (Young et al., 2005; Johnson et al., 2008), and more recently in non-human primates during early postnatal brain growth (Brambrink et al.; Olney et al., 2004; Zou et al., 2009). NMDAR blockade in the immature brain may also affect plasticity. For example, NMDAR signaling regulates NMDAR subunit expression (Wilson et al., 1998), and AMPAR incorporation at developing synapses (Shi et al., 1999; Hall et al., 2007; Wang and Kriegstein, 2008). The period of neuronal constitutive apoptosis also coincides with rapid brain growth and synaptogenesis.

Because memantine is clinically available, safe in adults (Thomas and Grossberg, 2009), and has a demonstrated target in the brain of preterm infants (Manning et al., 2008), we decided to investigate its safety profile in normal developing rat pups. Previously we reported that memantine did not alter gross myelination or cortical growth in uninjured immature rats (Manning et al., 2008). Here we compare memantine's effects on constitutive apoptosis to those of MK-801. Additionally, we evaluate short and longer-term effects of memantine on markers of synapse development in the immature rat brain, including NMDAR subunits, AMPAR subunits, Synapsin-1, and PSD95.

2. Materials and Methods

2.1. Animal procedures

Long Evans male rat pups (Charles River Laboratories) were maintained in a temperature-controlled animal care facility with a 12 h light/dark cycle. All procedures were approved and in accordance with guidelines of the Animal Care and Use Committee at Children's Hospital (Boston, MA) and the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. All efforts were made to minimize animal suffering and the number of animals used.

2.2. Pharmacologic treatment

A single dose schedule of memantine (Tocris Bioscience) treatment was used for comparison of constitutive apoptosis with MK-801 (Sigma): P6 rat pups received intraperitoneal (i.p.) injections of either memantine 20 mg/kg or 40 mg/kg, MK-801 1 mg/kg, or PBS vehicle. We used MK-801 as a positive control because its effects and dose response on apoptosis in the developing brain have been well documented (Ikonomidou et al., 1999), and it is most similar in action at the NMDAR to memantine. In addition we examined the effects of dosing following the regimen previously reported to result in neuroprotection (Manning et al., 2008): for TUNEL and Western blot experiments analyzing the effects of neuroprotective memantine, a loading dose of 20 mg/kg i.p. was given starting at P6, followed by three additional 1 mg/kg doses at 12 h intervals. All drug doses were prepared in a uniform 10 ml/kg volume of administration. The different drug treatments were distributed within each litter to avoid the potential variation that may arise between litters.

2.3. Histochemical analysis

Brains were collected at P7 for single dose memantine comparison to MK-801 TUNEL analysis, and at P10 for neuroprotective memantine treatment TUNEL analysis. For Western blots, after neuroprotective memantine treatment, brains were collected at P10 and P21.

For TUNEL immunohistochemistry, Brains were extracted and post-fixed overnight in 4% paraformaldehyde in PBS, followed by cryoprotection in 30% sucrose in PBS in preparation for sectioning. 16 μ m coronal cryostat sections (Leica CM3050 S) were collected on Superfrost Plus slides. For this analysis we examined the region of the mid-dorsal hippocampus which corresponds to the most affected area in our rat PVL model, and also

because it allows assessment of multiple brain regions: cortex, amygdala, caudate putamen, hippocampus, and thalamus. Furthermore, it is in the region of parietal cortex which in prior studies was most sensitive to the apoptotic effects of MK-801 (Ikonomidou et al., 1999). The stereotaxic coordinates were: for P10, 2.8–3.1 mm from bregma, 2.6–3.0 mm lateral to midline; for P21, 3.0–3.4 mm from bregma, 2.4–3.4 mm lateral to midline (Sherwood and Timiras, 1970). We chose TUNEL staining to assess neuronal injury, which has been used in the assessment of apoptosis with NMDAR antagonists (Ikonomidou et al., 1999). Other studies have used activated caspase 3 (AC3) immunohistochemistry to demonstrate drug-induced neuronal apoptosis which, while sensitive, is early and transient lasting just 6 h. Furthermore, AC3 is expressed in different neuronal populations at different times following injury (Olney et al., 2002). Where both techniques have been compared they have shown similar results (Ikonomidou et al., 1999; Olney et al., 2002). Thus TUNEL, a later and more robust marker of apoptosis, was more suited to our paradigm where different neuronal populations were examined 24 to 48 h after memantine dosing.

TUNEL staining (Roche) with Nickel-enhanced DAB peroxidase detection (Vectastain) was performed on slide-mounted sections that were lightly counterstained with methyl green 0.5% in 0.1 M NaAc pH 4.2, dehydrated through ethanol to HistoClear, and coverslipped in DPX mounting media. Images were obtained on a Zeiss Axioscope, using a Spot digital camera and Advanced 4.5 software (Diagnostic Instruments). Non-overlapping low power photomicrographs were taken of parietal cortex, hippocampus, thalamus, caudate-putamen, and amygdala. The total area examined for each animal varied by region from about 1.7 mm² for caudate putamen to over 4 mm² for thalamus. Photomicrographs were analyzed for TUNEL positive cells using imageJ (NIH) image-analysis software and represented graphically for each brain region as a ratio of memantine to PBS vehicle-treated TUNEL positive cells/mm².

For Western blot analysis, brain tissue was dissected out immediately after decapitation, and cerebral mantle (cortex and sub cortical white matter) was separated under a dissecting microscope. At this stage of brain development the sub cortical white matter is largely adherent to overlying cortex so cannot be reliably and accurately separated. Tissue was rapidly frozen in ethanol and stored at –80°C until used for protein extraction. Membrane protein samples from cerebral mantle were prepared as described previously (Wenthold et al., 1992; Talos et al., 2006B). Complete Mini Protease Inhibitor Cocktail Tablet (Roche) and phosphatase inhibitors PMSF (10 mM), Na-orthovanadate (10 mM), and okadaic acid (1 mM) were added to inhibit proteases and phosphatases. Total protein concentrations were measured using Bradford protein assay (Bio-Rad), and samples were diluted to obtain equal amounts of protein in each sample. Samples were electrophoretically separated on 7.5% Criterion Tris-HCl precast gels (Biorad) and transferred to polyvinylidene difluoride membranes. Blots were blocked (5% milk powder in TBST) and incubated with primary and secondary antibodies. Primary antibodies raised against NR1 1:1000 120 kD, NR2B1:500 180 kD, NR2A1:200 180 kD, GluR11:200 106 kD, GluR21:200 108 kD, Synapsin-11:200 80/77 kD, β -actin 42 kD (all Chemicon), PSD95 1:200 95 kD (Cell Signaling) were used. Anti-rabbit or mouse HRP secondary antibodies 10 μ g/ml (Pierce) were used at a dilution of 1:5000. Antibodies recognizing bands of similar sizes were used on different blots. Blots were probed no more than 4 times and were stripped with Restore Stripping Buffer (Pierce). Bands were detected with Super-West Femto Maximum Sensitivity Substrate reagent (Pierce) as described previously (Talos et al., 2006b). Digital images were recorded using the Fuji Image 3000 chemiluminescence detection system. Densitometric analyses of the digital images were performed using Fuji Film MultiGauge image-analysis software to measure the optical signal density from each sample. Densitometric values were normalized to β -actin, and expressed as a ratio of the mean PBS vehicle value.

2.4. Statistical analysis

Data were analyzed with Sigma Stat 3.11 software (Systat Software 2004) and expressed as mean $\pm$ SEM. Normally distributed data differences between two groups were compared using Student's *t* test. Multiple groups were compared using one-way ANOVA with the Bonferroni multiple-comparison *post hoc* test. Nonparametric data were analyzed using the Mann-Whitney rank sum test. A *P* value of 0.05 was considered statistically significant.

3. Results

3.1. Comparative effects of single dose treatment with memantine and MK-801 on density of TUNEL positive cells

TUNEL positive cells were assessed at P7 and were evident as darkly stained pyknotic nuclei within a background of healthy appearing cells counterstained with methyl green (Fig. 1). In PBS-treated control rats, the distribution of TUNEL positive cells was sparse (Fig. 1A), consistent with prior reports (Ikonomidou et al., 1999). Furthermore, 1 mg/kg MK-801 caused a significant increase in constitutive apoptosis in all brain regions examined (Fig. 2A–E) in a distribution as previously reported (Ikonomidou et al., 1999). For example, in cortex the majority of apoptotic cells were clustered in layer II (Fig. 1D). In contrast, memantine at the 20 mg/kg loading dose did not result in any increases in the level of constitutive apoptosis in any brain area compared to control (Fig. 2A–E). Twice the neuroprotective loading dose (40 mg/kg) also did not cause a statistically significant increase the level of TUNEL positivity in most brain regions analyzed, with the exception of the thalamus ($p = 0.004$) (Fig. 2E). We noted no change in motor behavior in the hour after the pups received memantine; however MK-801 resulted in a noticeable reduction in activity. To capture any significant biological effect that may interfere with our results we measured body weight, reflecting longer-term activity and nursing ability. No changes in body weight were observed in memantine treated rats, while MK-801 significantly reduced gain in weight compared to PBS treated control littermates (data not shown).

3.2. Neuroprotective dosing with memantine in the P6 rat does not increase constitutive apoptosis in the developing brain

Given the lack of adverse effect on constitutive apoptosis of the 20 mg/kg loading dose of memantine, we next evaluated the effect of doses of memantine that have previously been demonstrated to protect against brain injury (Manning et al., 2008). TUNEL staining was assessed at P10. Rats treated with memantine showed no increase in TUNEL positive cells compared to PBS treated control rats in all 5 regions examined: parietal cortex, amygdala, caudate putamen, hippocampus, or thalamus (Fig. 3). However there were sparse TUNEL positive cells seen in a variety of areas in both groups consistent with normal patterns of constitutive apoptosis (Ikonomidou et al., 1999; Bittigau et al., 2002). Body weight measured every 12 h showed no significant differences between the memantine and PBS groups (data not shown).

3.3. Effects of neuroprotective dosing with memantine on cortical expression of NMDAR and AMPAR subunits and the synaptic proteins Synapsin-1 and PSD95

Given the lack of effect of neuroprotective dosing on constitutive apoptosis in the developing brain, we next tested whether more subtle neurodevelopmental processes, such as glutamate receptor and synapse development, may be affected by the neuroprotective dosing regimen.

At P10 the neuroprotective memantine dosing schedule did not result in any change on Western blots in the cortical expression of the obligate NR1 subunit or in the early expressed NR2B subunit, compared to littermates given PBS. However this dosing did cause a 50%

increase in the NR2A subunit ($p = 0.003$) (Fig. 4A). No changes were detected in AMPAR subunits GluR1 and GluR2, or in the synaptic proteins Synapsin-1 and PSD95 at P10 (Fig. 4A).

At P21 NR1 and NR2B in memantine treated pups were unchanged from PBS control on Western blots. Notably, the early increase in NR2A seen at P10 was no longer evident at P21 (Fig. 4B). As at P10 there were no significant changes detected in levels of AMPAR subunits GluR1, GluR2, or in the synaptic proteins, Synapsin-1 and PSD95 at the later P21 time point (Fig. 4B).

4. Discussion

We show here that memantine does not increase constitutive apoptosis in the immature rat at neuroprotective dosing. Substantially higher dosing with memantine may increase constitutive apoptosis but still does not approach the pathologic effect of MK-801 at a pharmacologic dose commonly used in rodents. Thus, memantine appears safer than MK-801 with respect to constitutive apoptosis, but not without potential risk, since the specific relation between neuroprotective and toxic dose could differ in the human. Our additional data show that neuroprotective dosing with memantine increases cortical membrane levels of the NMDAR subunit NR2A at P10. However, we see no alteration in AMPAR subunit expression, or in the two synaptic proteins Synapsin-1 and PSD95.

4.1. Memantine and constitutive apoptosis

Here we show for the first time that memantine at neuroprotective doses does not alter constitutive apoptosis. This is consistent with *in vitro* studies of primary neuronal cultures or human neuroblastoma SH-SY5Y cells, where pharmacologically relevant doses of memantine (0.05–2.0 μM) did not cause cytotoxicity but did attenuate doxorubicin or staurosporine induced apoptosis in a developmentally specific manner (Jantas-Skotniczna et al., 2006; Jantas et al., 2008; Jantas and Lason, 2009). Interestingly however, this *in vitro* effect was NMDAR independent. Our *in vivo* result contrasts with a recent report by Liu et al. in which they showed a significant decrease in apoptosis in hippocampus CA1 and in sub cortical white matter in memantine treated rat pups (Liu et al., 2009). Here we also show that at the higher loading dose of 40 mg/kg, 40-fold higher than the effective maintenance dose, there is a trend towards increased apoptosis in all brain regions, reaching statistical significance in the thalamus. With our positive control, MK-801, *all* regions show statistically significant increases in apoptosis from about 3 to 7 fold, in a similar range to that previously reported (Ikonomidou et al., 1999). We sampled whole brain regions and not specific nuclei or cortical layers, so the inter-region differences in apoptosis may in part reflect the underlying neuronal density. However, regional differences in temporal response to pharmacologic induced neuroapoptosis could also play a part (Ikonomidou et al., 1999; Olney et al., 2002). Our result showing no change in apoptosis with neuroprotective memantine dosing compliments our prior data showing no effect on myelination, or the thickness of the cortex in immature rats (Manning et al., 2008). The apparent relative safety of memantine compared to other NMDAR antagonists may be explained by its fast on/off rate and strong voltage-dependency at the NMDAR favoring channel blockade only in pathological circumstances, e.g. excitotoxic injury (Chen et al., 1992; Chen et al., 1998; Lipton, 2007). One caveat is that our study was performed in normal animals. Pathological conditions in which memantine may be used, such as hypoxia-ischemia, may alter neuronal sensitivity to apoptosis. In practice, in our PVL model this is difficult to ascertain as the injured brain shows TUNEL positive neuronal cell death (unpublished data). It should be noted that in memantine treated animals this injury was lessened (Manning et al., 2008).

4.2. Memantine and NMDAR subunit expression

While a number of studies have focused on constitutive apoptosis with NMDAR antagonists, few have examined potential effects of these drugs on NMDAR subunit expression. The NMDAR is a heteromeric receptor made up of multiple subunits. Subunit composition regulates function, is cell-type specific, and is both developmentally regulated and experience-dependent (Yashiro and Philpot, 2008). NR1 is thought to be obligate to a functioning receptor. One major developmental subunit switch in neurons is from NR2B to a mixture of NR2B and NR2A (Williams et al., 1993). In the rat NR2B levels remain relatively constant in the postnatal period; in contrast NR2A is very low at birth, rising to adult levels in the first 3 post-natal weeks (Sheng et al., 1994; Yashiro and Philpot, 2008). Here we show the up regulation of cortical membrane-associated NR2A at P10, shortly after the end of the neuroprotective memantine dosing schedule. This increase, relative to NR1 and NR2B levels, is no longer seen at P21, and is within the approximately two-fold increase in NR2A between P10 and P21 previously reported in rat visual cortex (Quinlan et al., 1999). This result is also consistent with other studies of cortical expression of NR2A following MK-801 administration in neonatal rat brain (Wilson et al., 1998), or memantine administration in the adult brain (Rammes et al., 2001), although these studies only evaluated the acute response. This precocious switch to NR2A has been hypothesized to explain the paradoxical super sensitivity of neonatal rats to NMDA-mediated injury following MK-801 administration (McDonald et al., 1990; Wilson et al., 1998), a potential concern if memantine is used in preterm infants to prevent hypoxia-ischemia induced excitotoxic injury. Furthermore, an increase in NR2A could theoretically alter normal plasticity (Fagioli et al., 2003; Yashiro and Philpot, 2008; Cho et al., 2009). However, the present study shows that even the multi-day neuroprotective dosing regimen, which is clinically relevant, does not have long lasting effects on NMDAR subunit expression, consistent with our previous observation that there are no long term effects on cortical thickness (Manning et al., 2008).

4.3. Lack of effect of memantine on AMPAR subunit expression and synaptogenesis

Here we report for the first time, both acute and longer term effects of neuroprotective memantine dosing on markers of synapse development in the immature brain. The developmental switch from NR2B to NR2A enhances the incorporation of AMPARs into synapses (Shi et al., 1999; Hall et al., 2007). Thus, our finding of precocious NR2A expression suggests that memantine could potentially alter AMPAR expression. As with NMDARs, AMPAR subunit composition is developmentally regulated in rodents and humans (Talós et al., 2006b; Talós et al., 2006a). Our neuroprotective memantine dosing did not alter levels of GluR1 or GluR2 with respect to PBS treated control rats either early at P10 or later at P21. These findings are consistent with previous data showing that memantine does not block maze learning or long term potentiation in immature rat brain at the same concentrations used in our study (Chen et al., 1998).

Given the temporal relationship of our experimental paradigm to the brain growth spurt corresponding to developmental synaptogenesis (Dobbing and Sands, 1973; Olney et al., 2004), we also evaluated the effect of neuroprotective memantine dosing on levels of two proteins integral to active synapses, Synapsin-1 and PSD95. Neither Synapsin-1 nor PSD95 levels were altered by neuroprotective memantine dosing either early at P10 or later at P21.

4.4. Relevance to clinical neonatal practice

The clinically available NMDAR antagonist memantine attenuates brain injury in a rat model of PVL (Manning et al., 2008). However, the translation of this finding into the clinic has been uncertain, due to the fact that other NMDAR antagonists have been reported to increase constitutive apoptosis during early postnatal cerebral development. A recent

pediatric clinical application of memantine is intellectual developmental disorders, and there are ongoing studies evaluating a daily dose of 0.4 mg/kg in children 3–12 years of age with PDD (Owley et al., 2006; Chez et al., 2007). This is below the maintenance dosing of 1 mg/kg we use in our rat model, and the data we present here suggest that this is well within a safe range in the developing brain.

Arguably, the most concerning potential side effect of NMDAR antagonists to the developing brain is an increase in constitutive apoptosis. This is common to all NMDAR antagonists tested in immature rodents. A recent review details preliminary data suggesting that routine human neonatal anesthesia may result in cognitive disabilities (Istaphanous and Loepke, 2009). Olney has argued that the structural and cognitive effects of ethanol (an NMDAR antagonist and GABA (A) agonist) seen in Fetal Alcohol Spectrum Disorder (FASD), may in part be due to increased constitutive apoptosis. He suggests that FASD is a human example of the potential for toxicity of the NMDAR antagonists (and GABA-mimetics) (Olney et al., 2004). Despite this accumulating data, with no acceptable alternatives both drug classes are still used routinely in anesthesia for preterm and term infants. In summary this study supports the relative safety of memantine at neuroprotective doses in the immature rodent brain. However these findings do not preclude the possibility of changes in long term neuronal network function or whole animal behavior. Understanding the potential risks of therapies in suitable preclinical immature animal models should guide initial human clinical trials of those therapies in preterm infants and young children.

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Research Highlights

1. Memantine does not increase neuronal apoptosis in the rat brain at neuroprotective doses.
2. Memantine causes no long term changes in NMDAR, AMPAR subunits, or synaptic markers.
3. As memantine is currently in clinical trials in children, safety data is important.

Figure 1.

Representative photomicrographs of 16 µm coronal sections of parietal cortex 24 h after treatment with (A) PBS vehicle, (B) memantine 20 mg/kg, (C) memantine 40 mg/kg, (D) MK-8011 mg/kg. Insets magnified to show cortical layer II. TUNEL positive (black pyknotic) cells are most evident in MK-801 treated animals (D and inset). TUNEL negative cells are seen stained lightly with methyl green counterstain. Pictures were taken at low power to maximize scoring. Other regions examined were amygdala, caudate-putamen, hippocampus, and thalamus. Scale bar = 200 µm (inset scale bar = 600 µm).

Figure 2.

Single dosing effect of memantine compared to MK-801 on constitutive apoptosis. We analyzed the number of apoptotic cells per unit area in 5 anatomical brain regions (amygdala (A), caudate-putamen (B), parietal cortex (C), hippocampus (D), thalamus (E)), 24 h after a single dose of PBS (vehicle control), memantine (20 mg/kg or supra-pharmacologic 40 mg/kg), and MK-801 (1 mg/kg). Data are represented as a percent of the mean background level of apoptosis in PBS vehicle treated control rats. Memantine at neuroprotective loading dose (20 mg/kg) does not increase apoptosis relative to control PBS. Memantine at twice neuroprotective loading dose (40 mg/kg) increases apoptosis in all regions, but this only reaches statistical significance in the thalamus $P = 0.004$. In contrast, a single pharmacologic dose of MK-801 causes a significant increase in apoptosis approximately 3–6 fold control levels in all areas: amygdala $P = 0.001$, caudate-putamen $P = 0.001$, parietal cortex $P = 0.021$, hippocampus $P = 0.001$, thalamus $P = 0.004$. (One-way ANOVA, $n = 10$ PBS, $n = 10$ memantine 20 mg/kg, $n = 8$ memantine 40 mg/kg, $n = 9$ MK-801).

Figure 3.

Neuroprotective dosing effect of memantine on constitutive apoptosis. Memantine (20 mg/kg loading, 1 mg/kg q 12 h $\times$ 3) sufficient to prevent hypoxic-ischemic injury in the PVL model, or PBS vehicle, was administered to P6–8 rat pups, and brains were collected at P10. We analyzed the number of apoptotic cells per unit area in the same 5 anatomical brain regions detailed in the single dosing experiment. Data are represented as a percent of the mean background level of apoptosis in PBS vehicle treated control rats. Memantine neuroprotective dosing does not significantly alter levels of constitutive apoptosis in any of the 5 brain regions examined: parietal cortex $P = 0.678$, amygdala $P = 0.198$, caudate-putamen $P = 0.879$, hippocampus $P = 0.844$, thalamus $P = 0.678$ (Rank Sum test: $n = 15$ PBS, $n = 14$ memantine).

Figure 4.

Short and longer-term evaluation of cortical membrane protein NMDAR subunit, AMPAR subunit, and synaptic protein (Synapsin-1, PSD95) levels following neuroprotective memantine dosing in P6–8 rat pups. Representative Western blots are shown for each protein, paired with β -actin immediately below, in memantine treated (upper pair) and vehicle treated (lower pair) animals. (A) Western blot data of brain cortex membrane protein at P10 shows a significant increase in the mature NMDAR-subunit NR2A compared to PBS vehicle-treated rats (Student *t* test, $n = 15$ PBS, $n = 17$ memantine: $P = 0.003$). The obligate NMDAR subunit NR1 and the early expressed NR2B subunit are not similarly increased. There are no significant alterations in AMPAR subunits GluR1 or GluR2. The synaptic proteins Synapsin-1 and PSD95 similarly are not altered by the memantine treatment protocol. (B) Western blot data at P21 shows no longer-term alterations in NMDAR subunits (NR1, NR2A, NR2B), AMPAR subunits (GluR1, GluR2), or synaptic proteins (Synapsin-1, PSD95).