Exp Brain Res (2010) 201:479–488
DOI 10.1007/s00221-009-2059-z

RESEARCH ARTICLE

Postnatal exposure to MK801 induces selective changes in GAD67 or parvalbumin

Christopher Paul Turner · Danielle DeBenedetto · Emily Ware · Robert Stowe · Andrew Lee · John Swanson · Caroline Walburg · Alexandra Lambert · Melissa Lyle · Priyanka Desai · Chun Liu

Received: 14 April 2009 / Accepted: 14 October 2009 / Published online: 3 November 2009

Abstract Brain injury during the last trimester to the first 1–4 years in humans is now thought to trigger an array of intellectual and emotional problems later in life, including disorders such as schizophrenia. In adult schizophrenic brains, there is a specific loss of neurons that co-express glutamic acid decarboxylase-parvalbumin (GAD67-PV). Loss of this phenotype is thought to occur in mature animals previously exposed to *N*-methyl-D-aspartate receptor (NMDAR) antagonists during late gestation or at postnatal day 7 (P7). However, in similarly treated animals, we have previously shown that GAD67 and PV are unaltered in the first 24 h. To more precisely define when changes in these markers first occur, we exposed rat pups (P7 or P6–P10) to the NMDAR antagonist MK801 and at P11 co-stained brain sections for GAD67 or PV. In the cingulate cortex, we found evidence for a reduction in PV (GAD67 levels were very low to undetectable). In contrast, in the somatosensory cortex, we found that expression of GAD67 was reduced, but PV remained stable. Further, repeated but not single doses of MK801 were necessary to see such changes. Thus, depending on the region, NMDAR antagonism appears to influence expression of PV or GAD67, but not both. These observations could not have been predicted by previous studies and raise important questions as to how the GAD67-PV phenotype is lost once animals reach maturity. More importantly, such differential effects may be of great clinical importance, given that cognitive deficits are seen in children exposed to anesthetics that act by blocking the NMDAR.

Keywords Rat · Neonatal · Dizocilpine · GABA · Glutamate · Calcium-binding protein

C. P. Turner (✉) · D. DeBenedetto · E. Ware · R. Stowe · A. Lee · J. Swanson · C. Walburg · A. Lambert · M. Lyle · P. Desai · C. Liu
Neurobiology and Anatomy,
Wake Forest University School of Medicine,
1 Medical Center Blvd, Winston-Salem,
NC 27157-1010, USA
e-mail: cpturner@wfubmc.edu

Introduction

Schizophrenia is a disorder increasingly associated with developmental perturbations (Lewis and Levitt 2002; Remschmidt 2002; Eastwood 2004). Animal models that test sensorimotor or cognitive changes in adults previously exposed to *N*-methyl-D-aspartate receptor (NMDAR) antagonists during the first week or so of postnatal life describe deficits reminiscent of those observed in schizophrenics (Wang et al. 2001; Harris et al. 2003; Wang et al. 2003). Further, schizophrenia is associated with a number of key pathologies that include loss of glutamate function (Olney and Farber 1995; Carlsson et al. 1999; Kristiansen et al. 2007), changes in γ-amino butyric acid (GABA)-ergic transmission (Beasley et al. 2002; Lewis and Moghaddam 2006; Hashimoto et al. 2008) and loss of the glutamic acid decarboxylase-parvalbumin (GAD67-PV) neuronal phenotype (Hashimoto et al. 2003). Many general anesthetics (that act by blocking the NMDAR) are now known to cause cognitive deficits in young adults previously exposed to such agents between the ages of 0–4 years (Wilder et al. 2009). Thus, animal models that use glutamate dysfunction to induce age-dependent brain injury may not only provide insight into why the GAD67-PV phenotype is lost in clinical syndromes such as schizophrenia, but may also allow us to ask if exposure to general anesthetics at very young ages promote similar pathologies.

The early postnatal period in rodents is thought to be roughly equivalent to the last trimester/first 1–4 years of infant life in humans (Rice and Barone 2000) making this period in rats ideal for modeling brain injury in very young children. For example, when postnatal day 7 (P7) rats are exposed to NMDAR antagonists, apoptotic injury is induced within hours (Ikonomidou et al. 1999; Wang et al. 2001; Turner et al. 2002, 2007; Jevtovic-Todorovic et al. 2003; Dzietko et al. 2004; Wang et al. 2004). However, sensitivity to such agents diminishes rapidly as animals mature. These animal studies suggest that children may be at risk when exposed to agents that act through the NMDAR (Ikonomidou et al. 1999; Turner et al. 2002, 2007). For example, age-dependent apoptosis is observed throughout the forebrain following exposure to the general anesthetic ketamine, to anti-epileptics or other NMDAR antagonists (Jevtovic-Todorovic et al. 2000; Beals et al. 2003; Fredriksson et al. 2007; Ikonomidou et al. 2007; Slikker et al. 2007; Kaindl et al. 2008; Stefovska et al. 2008; Bercker et al. 2009; Lunardi et al. 2009). Further, a recent study suggests that the consequences of anesthesia-induced injury may have profound clinical implications (Wilder et al. 2009).

Although there has been much focus on potential mechanisms and short-term molecular changes, there are also long-term outcomes that suggest permanent damage may have occurred (Kaindl et al. 2008). More specifically, neurons that express both the calcium-binding protein (CaBP) PV and the GABAergic marker, glutamic acid decarboxylase 67 (GAD67), are thought to be lost following exposure to MK801 or phencyclidine during the perinatal period (Abekawa et al. 2007; Wang et al. 2007). We have previously shown that expression of CaBPs in general, and PV in particular, increases abruptly at a time when sensitivity to NMDAR blockade rapidly declines (Lema Tomé et al. 2007). Similarly, GAD67 is low at or around P7 and only reaches adult levels relatively late in postnatal life (Greif et al. 1991, 1992; Golshani et al. 1997; Guo et al. 1997; Jiao et al. 2006; Yin and Tan 2007; Turner et al. 2009c). Further, at P7, induction of the pro-apoptotic marker, activated caspase-3, does not overlap with expression of CaBPs (Lema Tomé et al. 2006a, b) or GAD67 (Turner et al. 2009b). Importantly, in these same studies, we have found no evidence for decreased expression of PV or GAD67 within the first 24 h. It is likely then that antagonist-induced loss of the GAD67-PV phenotype (Abekawa et al. 2007; Wang et al. 2007) is secondary to the cell death observed immediately after NMDAR blockade, but exactly when such changes first occur is presently unknown.

To determine if antagonism of the NMDAR can lead to changes beyond the first 24 h following the initial receptor blockade, we exposed neonatal rats to vehicle or MK801 from P6 to P10, and at P11 examined the expression of proteins associated with GABAergic transmission (GAD67) or buffering of intracellular calcium (PV). Although others have examined short-term and long-term changes using the microarray approach (Kaindl et al. 2008), this technique cannot reveal what changes may take place at the histological level. Thus, using immunohistochemistry (IHC), we focused on the cingulate cortex (Cg) because other groups report that it is in the midline cortical structures of mature animals that GAD67-PV changes are observed (Abekawa et al. 2007; Wang et al. 2007). We also included the anterior somatosensory cortex (SSC_{ANT}) in this study because we have previously observed that the GAD67-positive puncta of layer IV in this region display a close association with cells undergoing apoptosis 8 h after MK801 exposure (Turner et al. 2009b). The findings we describe are discussed in the context of postnatal development, as well as with respect to the long-term effects of general anesthetics on very young children.

Methods

All in vivo procedures used in these studies were approved by the Wake Forest University Animal Care and Use Committee and were in compliance with NIH guidelines. All chemicals used in these studies were from Sigma-Aldrich (St Louis, MO), unless otherwise stated.

Treatment groups, injections and perfusions

A single injection of MK801 (dizocilpine; 1 mg/kg) at P7 has been shown to induce robust and widespread injury throughout the forebrain (Ikonomidou et al. 1999; Turner et al. 2002, 2007). However, it was unclear if this would be sufficient to induce lasting changes in downstream events following NMDAR blockade-induced triggering of apoptosis. Thus, we treated rat pups with a single injection of MK801 at P7 (1MK; $N = 4$) or repeated injections at P6, P8 and P10 (3MK; 3 injections total; $N = 6$). In addition to increasing the exposure to MK801 over a longer period, this three-dose strategy allowed us to compare outcomes with long-term studies conducted in this laboratory using the same approach (Lyall et al. 2009). As controls, we injected another group with vehicle at P6, P8 and P10 (vehicle; 3 injections total; sterile PBS, pH 7.4; $N = 6$). The vehicle and 3MK groups were represented across two litters. In the current study, our experimental design included animals from only one litter for the 1MK group, as pilot studies using the same injection protocol suggested we would not see changes in expression of GAD67 or PV after only one exposure. In all groups, pups had equal numbers of both genders. At P11, animals were anesthetized with 2% isoflurane and perfused with PBS, followed by 4%

paraformaldehyde in PBS (4% PFA). Brains were further fixed in 4% PFA at 4°C for another 24 h.

Immunohistochemistry

PFA-fixed brains (see above) were equilibrated in 10, 20 and 30% sucrose-PBS (3–4 days, total time) and cut frozen on a sliding microtome into 60 µm coronal sections, which were stored in PBS in 24-well plates. Within each 24-well plate, sections from each brain were placed in consecutive wells along a given row (6 wells total) so that (within each well) sections were 6 × 60 µm apart from the preceding section.

We first exposed brain sections to mouse monoclonal anti-GAD67 primary antibody (1:500; Chemicon, Temecula, CA) followed by AlexaFluor 594, donkey anti-mouse secondary antibody (1:200; Millipore, Eugene, OR). After PBS washes, sections were then exposed to a rabbit polyclonal anti-PV primary antibody (1:2,000; Swant, Switzerland), followed by AlexaFluor 488, goat anti-rabbit secondary antibody (1:200, Millipore). All incubations were performed in IHC buffer (1% BS-A, 0.1% TX100, PBS; pH 7.4). Exposure to primary antibodies was for 24 h at 4°C and to secondary antibodies for 2 h at room temperature. After PBS washes, sections were mounted onto glass slides (SuperFrost Plus; Fisher, Pittsburgh, PA), air-dried and coverslipped using VectorShield mounting media (Vector Labs, Burlingame, CA). Primary antibody omission served as an assay control and eliminated all specific staining. Specific staining for both GAD67 and PV were very consistent with previous publications that examined the histological distribution of these markers (Greif et al. 1991; Guo et al. 1997; Jiao et al. 2006; Yin and Tan 2007; Turner et al. 2009b, c).

Imaging and quantification

Under UV light, images were captured at 10×, 20×, 40× or 60× magnification (as needed) using an Olympus IX70 inverted fluorescent microscope (Olympus, Melville, NY), an Orca 238 digital camera (Hamamatsu, Bridgewater, NJ) and IPLab software (v3.65a, Scanalytics, Billerica, MA). Images were either processed in Adobe Photoshop for pseudo-color conversion for figure display or imported into ImagePro 5.0 (Media Cybernetics, Baltimore, MD) for semi-quantitative analysis (see below).

PV cell counts

As previously described (Lema Tomé et al. 2006a), we counted the number of PV-positive cells within the cingulate and somatosensory cortex from 20× images. Because these counts were not performed stereologically, we used very conservative parameters to estimate the number of PV cells in a given brain region. Briefly, within the count box (250 × 250 µm), we set the size and intensity thresholds to exclude background or artifact labeling (size was set to exclude anything <5 µm or >100 µm; intensity set to exclude any object with a value of <50 (twice background value), using a scale of 0 = no signal, 255 = highest intensity). Once these parameters had been set, counting was performed automatically by ImagePro 5.0, independent of the observer (counting performed by all authors except CPT). The number of positive PV profiles was expressed as cells per mm^2. We counted within layer V of the Cg, and within layer V of the anterior part of the somatosensory cortex (SSC_{ANT}; from approximately the frontal cortex to the decussation of the anterior commissure), according to Paxinos (2004). For both regions, we sampled from 6 to 8 sections per animal.

GAD67-ir line scans

Images of the SSC_{ANT} were captured at 10× and imported into Image Pro 5.0. Most sections required two such images to cover the full dorsomedial–ventrolateral extent of the SSC_{ANT}. In each image, a line was traced so as to pass through the central core of each GAD67-immunoreactive patch within layer IV of the SSC_{ANT}. Based on the gray scale values obtained (0–255, 0 being lowest intensity and 255 highest intensity), we found that each scan easily discriminated adjacent patches as a series of peaks and troughs. After background subtraction, we derived two sets of data from these gray scale values: (1) the number of GAD67 patches (peaks) per unit pixel length; (2) the intensity of each patch (peak pixel intensity). Data were expressed as mean peaks/length or mean intensity for each treatment group. We sampled from six to eight sections per animal.

Statistical analysis

Mean (± SE) of PV cells/mm^2, peaks/length or peak intensity was estimated for each treatment group (see above) and differences between the groups determined by a one-way ANOVA, using a Bonferroni post-test comparison of means. All statistical analyses were performed using GraphPad Prism, version 4.0 (GraphPad, San Diego, CA).

Results

Previous work from this laboratory has shown that neither PV nor GAD67 levels are altered in the first 8–16 h after MK801 exposure at P7 (Lema Tomé et al. 2006a, b; Turner et al. 2009b). However, in mature animals, others have

shown that there is a specific loss of PV expression (in a subpopulation of GAD67 neurons) in animals exposed to NMDAR antagonists at P7 (Abekawa et al. 2007; Wang et al. 2007). Collectively, these studies suggest that changes in GAD67 and/or PV must take place after P7, but before the animals mature. To understand more clearly when such changes first take place, we injected P7 rat pups with vehicle (sterile PBS; vehicle) or the NMDAR antagonist MK801. For comparison, single MK801 injections at P7 (1MK) were compared with three injections of MK801 spread over P6, P8 and P10 (3MK). At P11, we examined coronal brain sections for expression of GAD67 and PV in the Cg and the SSC_{ANT}.

General patterns of GAD67/PV expression

We first established overall expression patterns for the two proteins of interest. Thus, in Fig. 1, we present montages of 4× images that give a global view of GAD67 and PV expression in the anterior forebrain. Although data in Fig. 1 compare a vehicle- with a 3MK-treated animal, observations made with respect to all treatment groups are presented in greater detail in Figs. 3 and 4.

In the Cg, although GAD67-ir was detectable, it was very weak compared to other regions (Fig. 1a; see also Fig. 3a–c). In contrast, PV expression was robust in both cell bodies and processes (Figs. 1a, 3a–c). In related studies, we have shown that in the Cg region, somatic GAD67-ir at P7 is either observed in very few cells or not detectable at all (Turner et al. 2009c). In the SSC_{ANT}, GAD67-ir was observed as distinct patches within layer IV, whereas PV was found mostly in layer V (Figs. 1a, 2a). These patches appeared to be rich in GAD67-positive puncta (Fig. 2b, d) in agreement with earlier studies (Turner et al. 2009b). Although somatic GAD67 staining was present in the more superficial layers (I–III), it was often very weak compared to the immunoreactivity observed in layer IV (see Fig. 2a, b). However, clear somatic GAD67-ir was found in the deeper layers, in particular layer VI (see Fig. 2a, c). Regardless of staining intensity or distribution, few cells in the SSC_{ANT} were found to express both GAD67 and PV (Fig. 2b). Indeed, in any given territory that had somatic staining for both proteins, a distinctly non-overlapping distribution was typically observed (Fig. 2c).

A complete survey of the entire forebrain would be beyond the immediate scope of this current study and a more comprehensive analysis of postnatal GAD67 or PV expression can be found elsewhere (Lema Tomé et al. 2007, 2008; Turner et al. 2009b, c). However, some staining patterns for GAD67 and PV were worthy of note. For example, GAD67-ir was intense in the septum, the islands of Calleja, olfactory tubercle (Fig. 1a) and the thalamic reticular nucleus (TRN; Fig. 2e–g). Overlapping expression with PV was found in numerous subcortical structures, particularly in the TRN, where levels and distribution for both markers were closely matched (Fig. 2e–g). In contrast, in the adjacent globus pallidus, GAD67 expression was robust, but PV low to absent. Clearly, postnatal regulation of both markers differs substantially depending on the brain region.

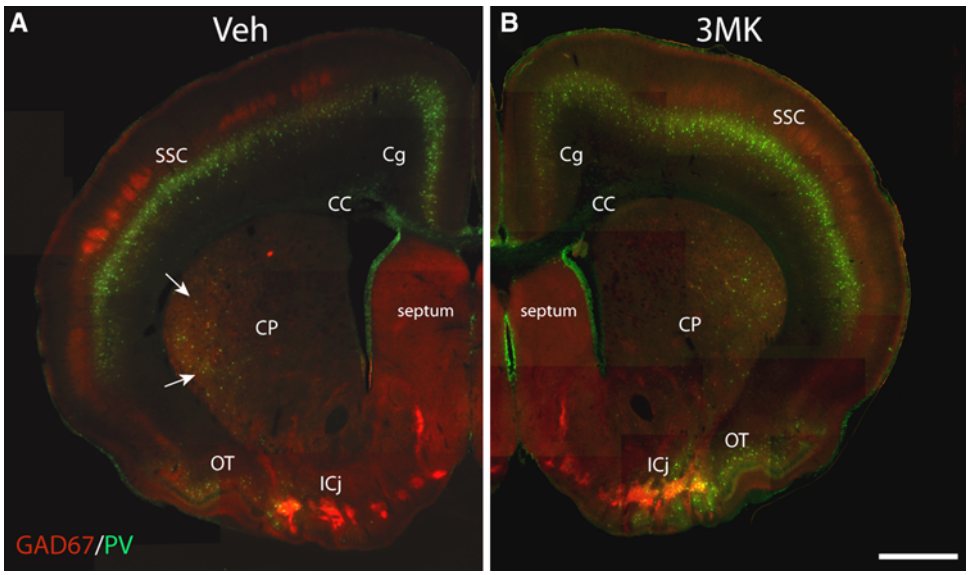

Fig. 1 Gross distribution of GAD67 and PV expression. Rat pups were injected with vehicle at P6, P8, and P10 (Veh; $N = 6$), or three single injections of MK801 at P6, P8 and P10 (3MK; $N = 6$) and allowed to recover until P11. At P11, brains were processed for expression of GAD67 and PV (see "Methods"). Montage of low-power images from the anterior forebrain of **a** vehicle or **b** 3MK-treated animal. *SSC* Somatosensory cortex, *Cg* cingulate cortex, *CC* corpus callosum, *CP* caudate putamen, *OT* olfactory tubercle, *ICj* islands of Calleja. *Arrows* in **a** point to PV-ir in the lateral CP. *Scale bar* is 1 mm

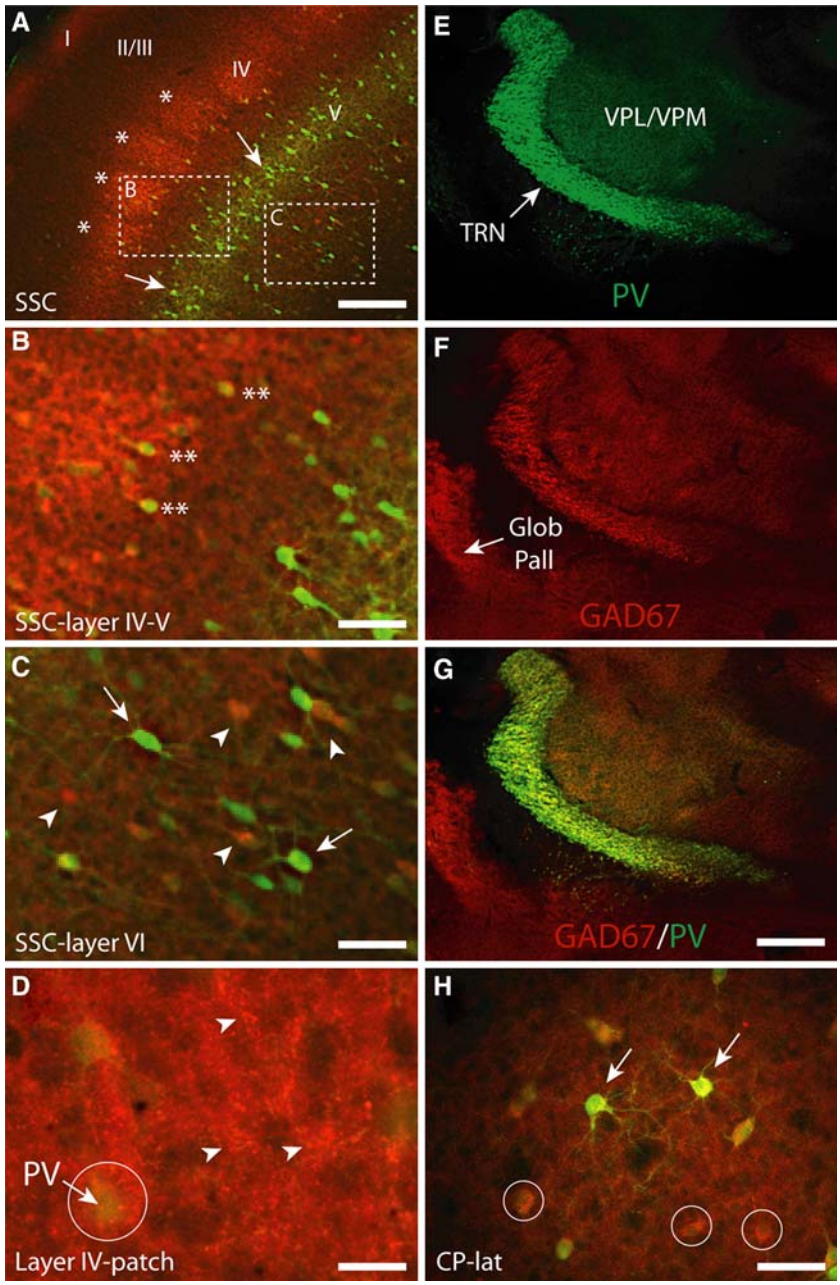

Fig. 2 Local distribution of GAD67 and PV. Brain sections from vehicle-treated animals stained for GAD67 and PV. **a** Expression of GAD67 in layer IV (*asterisks*) and PV in layer V (*arrows*) in the SSC_{ANT}. **b** Isolated PV-GAD67 cells were located at the base of each barrel field zone (see *double asterisks*). **c** In layer VI, numerous cells positive for either GAD67 (*arrowheads*) or PV (*arrows*) were found in the same territory (co-expression in the same cell not observed). **d** High power image of the central core of a single GAD67-rich patch (*arrowheads* GAD67-positive puncta; *circle* weakly stained PV neuron surrounded by GAD67-positive puncta). **e** PV expression in the thalamic reticular nucleus (*TRN*). **f** Expression of GAD67 in the same section. **g** Merged images of **e**, **f**. **h** High power view from the lateral edge of the caudate putamen (*CP*). *Circles* indicate GAD67-positive cells negative for PV; *arrows* point to PV-positive cells that appear to be also positive for GAD67. *Scale bars* as follows: **a** 200 μm, **b** 100 μm, **c** and **h** 50 μm, **d** 25 μm, **g** 200 μm. *Glob Pall* Globus pallidus

Although some of the merged images we have shown suggest that subpopulations of neurons appear to express both GAD67 and PV, the very weak staining for GAD67 in certain brain regions/layers prevented definitive assessment. Also, it must also be stressed that any assessment of the co-expression of both markers would require more rigorous confocal analysis and we bring attention to the overlapping nature of the expression patterns for both proteins (for example in the layer IV of the SSC_{ANT} or in the TRN) to contrast with the relative lack of overlap found elsewhere in most other forebrain regions.

MK801-induced changes in the cingulate cortex

A loss of the GAD67-PV phenotype in midline cortical regions has been reported by other groups (Abekawa et al. 2007; Wang et al. 2007), and so we next studied whether MK801 could induce changes in expression of these markers within the Cg. However, at P11 GAD67-positive cells were scarce and weakly labeled (see above), and so we focused on quantifying changes only in PV expression. To cover the entire dorsal–ventral extent of the Cg, we divided this region into Cg1, Cg1/Cg2 and Cg2 subregions

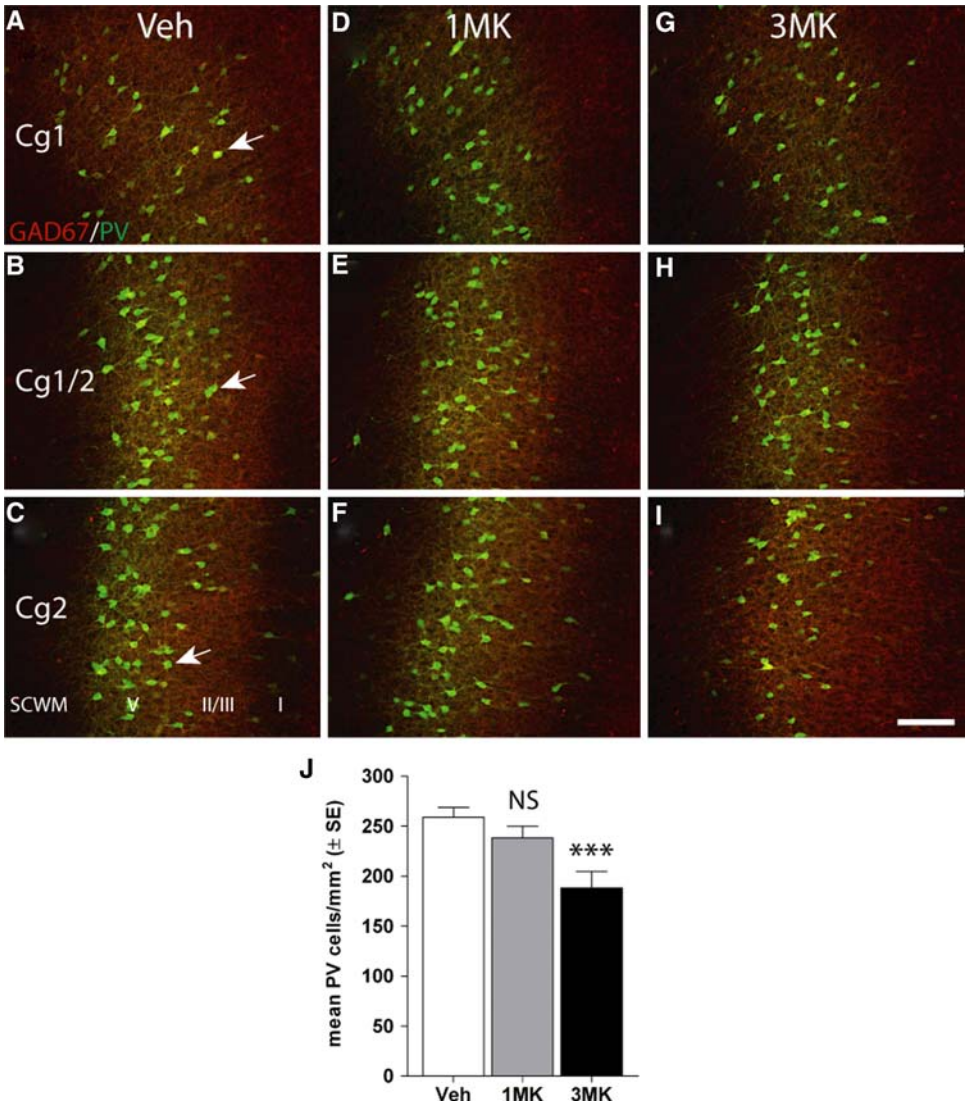

Fig. 3 Selective loss of PV in the cingulate cortex. Rat pups were injected and brain tissue processed as described in Fig. 1. PV numbers were estimated from three subregions: *Cg1*, *Cg1/2* and *Cg2* in **a**–**c** vehicle controls, **d**–**f** 1MK-treated animals or **g**–**i** 3MK-treated animals. **j** Combined counts (mean PV cells per $mm^2 \pm$ SE) from all three zones for vehicle, 1MK and 3MK groups; ***$P < 0.001$, *NS* not significant (ANOVA; Bonferroni post-test comparison of means). *Arrows* indicate PV-positive cells; scale in *F* is 100 μm. Note: these sections were also stained for GAD67-ir, but labeling was generally very weak or undetectable

(see Fig. 3). Compared to vehicle controls (Fig. 3a–c), although no changes were observed in the 1MK group (Fig. 3d–f), all subregions showed an apparent loss of PV cells in the 3MK group (Fig. 3g–i). When we quantified these changes, we found that 3MK-treated animals had 25% less PV numbers than those found in the vehicle-treated group (Fig. 3j; ***$P < 0.001$, ANOVA).

MK801-induced changes in the somatosensory cortex

We next examined changes in GAD67 and PV expression in the SSC_{ANT}. First, we determined the number and intensity of GAD67 patches in layer IV (see "Methods"). We found that in the sections from vehicle-treated animals, numerous distinct patches were quite evident, many of which were of high intensity (Fig. 4a; see also Figs. 1a, 2a). Although in the 1MK group, GAD67 appeared similarly distributed (Fig. 4b), in sections from 3MK-treated animals, both the number and the intensity of these GAD67-positive patches were diminished and in some sections undetectable (Fig. 4c; compare also Figs. 1a with 1b). When we quantified the mean number of peaks per unit length (see "Methods"), there were approximately 60% fewer peaks in the 3MK group compared to the vehicle (Fig. 4d; ***$P < 0.001$, ANOVA). Likewise, when we quantified patch intensity (see "Methods"), we found that the remaining patches in the 3MK group were 30% less intense than that found for vehicle (Fig. 4d; ***$P < 0.001$; ANOVA). No significant change was observed in the 1MK group compared to the vehicle controls.

We next studied if MK801 treatment could alter PV expression in the same brain region. As described earlier for the Cg, we noted a band of PV cells in layer V and so we estimated the mean density of PV cells from this layer in each group (see "Methods"). We did not observe any changes in the number of PV cells in 1MK or 3MK-treated

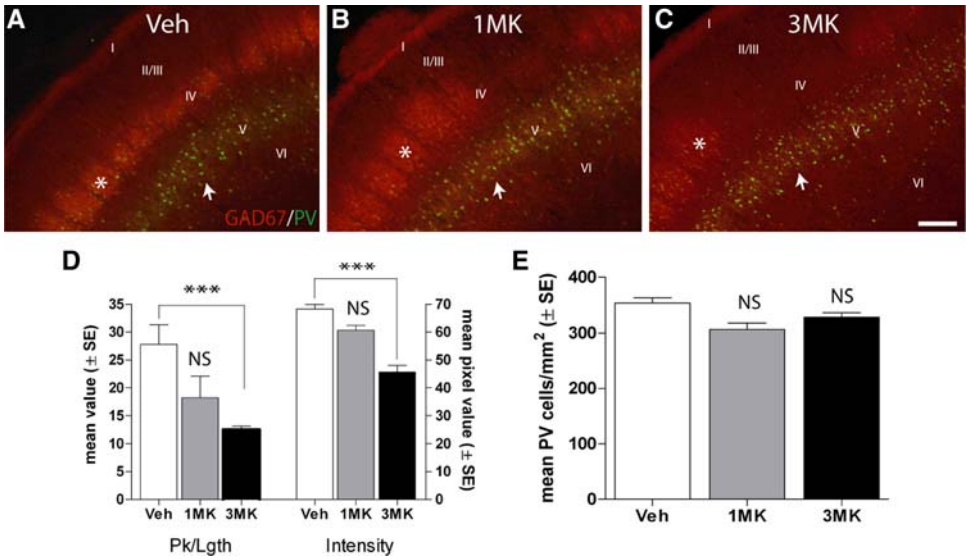

Fig. 4 Selective loss of GAD67 in the somatosensory cortex. Rat pups were injected and brain tissue processed as described in Fig. 1. **a** SSC_{ANT} of vehicle-treated animal. *Asterisk* indicates a single GAD67-rich patch in layer IV (approximately 12 are present in this one frame). *Arrow* indicates a single PV-positive cell in layer V. **b** SSC_{ANT} of 3MK-treated animal. **c** SSC_{ANT} of 3MK-treated animal. Note both the reduction in number and intensity of GAD67-rich patches in layer IV. **d** Quantification of the number of patches per unit pixel length and patch intensity (see "Methods"). **e** Quantification of PV cell number in layer V (see "Methods"). For both **d**, **e**: ***$P < 0.001$, *NS* not significant (ANOVA, Bonferroni post-test comparison of means)

animals, compared to vehicle controls (Fig. 4e). Thus, in contrast to GAD67 in this brain region, we can conclude that even with multiple MK801 injections, there are no changes in expression of this CaBP within days after the initial receptor blockade.

Discussion

The molecular changes that occur in the first 24 h after NMDAR blockade in P7 rats are well documented (Ikonomidou et al. 1999; Wang et al. 1999, 2004; Lema Tomé et al. 2006a, b; Lu et al. 2006; Turner et al. 2007; Wang and Johnson 2007; Kaindl et al. 2008; Ringler et al. 2008). Likewise, long-term, molecular, histological and behavioral changes have also been identified (Jevtovic-Todorovic et al. 2003; Abekawa et al. 2007; Wang et al. 2007; Kaindl et al. 2008; Lyall et al. 2009). However, we still do not have a clear picture of events taking place beyond the first 24 h after the initial blockade. In this study, therefore, we examined the influence of single or multiple episodes of NMDAR antagonism from P6 to P10 on expression of the GABAergic marker, GAD67, and the CaBP, PV, in P11 animals.

MK801-induced loss of PV

Our initial focus was on the Cg, as others have shown loss of PV in midline cortical regions following perinatal exposure to NMDAR antagonists (Abekawa et al. 2007; Wang et al. 2007). In our current study, whereas a single, transient loss of NMDAR activity (1MK group) was insufficient to change PV expression, repeated loss of receptor activity over a 4-day period (3MK group) triggered a decline in this CaBP. This may simply reflect a dosage effect, though it is only at the end of the P6–P10 period that PV expression emerges (Lema Tomé et al. 2007, 2008), suggesting that (in the present study) the timing of MK801 exposure rather than dosage may be a factor. However, given that prenatal exposure to MK801 can also trigger loss of GAD67-PV expression, it may be that ongoing developmental programs that precede the emergence of PV expression are sensitive to loss of NMDAR activity at many stages (in the Cg, at least). Perhaps, only after PV expression is fully established are such neurons safe from agents that block this receptor (Lema Tomé et al. 2006a, b, 2007, 2008). Indeed, when we simulate artificial buffering of calcium in the face of MK801 challenge, apoptosis is reduced or eliminated (Turner et al. 2009a).

MK801-induced loss of GAD67

In contrast to the relative absence in the Cg, GAD67-ir was easily detectable in the SSC_{ANT}, being particularly intense within the barrel fields of layer IV. However, unlike GAD67, PV expression in the SSC_{ANT} was quite similar in intensity and distribution to that found in the Cg. Repeated exposure to MK801 led to loss of GAD67 in the barrel field

region (layer IV of the $SSAC_{ANT}$), but there was no change in the number of PV-positive cells in layer V. Thus, in contrast to the Cg, expression of GAD67 in the SSC_{ANT} was sensitive to NMDAR blockade, whereas PV remained stable, suggesting that MK801-induced changes in these markers occur independently of each other. Although MK801 may reduce expression of the enzyme responsible for GABA production, its action on the GABAergic system as a whole may be more complex. For example, newborn rats exposed to this agent do not display changes in $GABA_A$ receptor expression (Penschuck et al. 1999). However, consistent with our studies, diminished barrel field development was observed, in agreement with others (Mitrovic et al. 1996).

In rodents, a critical source of sensory input to the CNS is the pathway projecting from the vibrissae of the whisker pad up to the barrel fields of the somatosensory cortex. This pathway plays a significant role in texture discrimination, navigation and exploration (Waite 2004), and its disruption can deprive animals of important information about their environment. Whereas primates do not have a whisker pad–barrel field system, damage to the equivalent somatosensory pathway or structures would nevertheless create aberrations in somatosensory processing, potentially resulting in clinically relevant cognitive or behavioral deficits at later ages.

Direct or indirect action of MK801?

Prior studies show that expression of GAD67 or PV is generally low to absent before P7, and adult-like levels and patterns appear no earlier than P10–P14, or much later depending on the brain region (Greif et al. 1991, 1992; Golshani et al. 1997; Guo et al. 1997; Jiao et al. 2006; Lema Tomé et al. 2007, 2008; Yin and Tan 2007; Turner et al. 2009c). Thus, at the post-translational level at least, MK801 has little substrate to act upon during the period leading up to P10 and beyond. It is possible that MK801 or similar agents may act at the transcriptional or translational level, though evidence supporting such a mechanism is yet to be presented. Implicitly then, the effect of NMDAR blockade on GAD67 or PV expression during the perinatal period has to be indirect. In support of this, MK801-induced expression of the pro-apoptotic marker activated caspase-3 does not occur in either PV (Lema Tomé et al. 2006a) or GAD67 soma (Turner et al. 2009b). Further, in both these studies, we see no evidence of any decline in these two markers in the first 24 h. Further still, loss of both markers in the same cell population is observed only at adult ages (Abekawa et al. 2007; Wang et al. 2007), suggesting that the pathology underlying the disappearance of the GAD67-PV phenotype is progressive in nature.

It could be argued that we have not considered what the effect(s) of a single injection of MK801 at P11 might have had on PV or GAD67 expression (in comparison to that found following a single injection at P7 or multiple injections from P6 to P10). The injection strategies used in this study were based on optimizing administration of MK801 at or around the period of peak cell death (P6–P10), as well to be consistent with previous studies from our laboratory that target long-term consequences of NMDAR blockade using a similar approach (Lyall et al. 2009). Thus, the present study was not designed to address effects of MK801 with respect to: (1) window of sensitivity; (2) later emergence of GAD67 or PV; (3) sensitivity of ongoing developmental programs that may influence later expression of these markers. However, future studies that more comprehensively explores how NMDAR blockade at different ages influences expression of GAD67 or PV could generate critical data that link the earlier events we have described here and elsewhere (Turner et al. 2009b, c) with loss of the GAD67-PV phenotype in adult animals (Abekawa et al. 2007; Wang et al. 2007).

Biological and clinical implications

Studies from this laboratory suggest that even in the absence of overt cell death, more subtle effects on growth cone activity, axon elongation and navigation, as well as dendritic branching are observed following MK801 exposure (Ringler et al. 2008). Thus, NMDAR blockade during the developmentally dynamic postnatal period can have a profound effect on neuronal survival, maturation and differentiation. It should also be noted that multiple MK801 administrations over the P6–P10 period has been used to examine changes in neurogenesis at later ages (Smith et al. 2007; Stefovska et al. 2008). In both studies, a clear loss of mitotic activity was noted in a number of forebrain regions. Clearly, NMDAR blockade at or around P7 leads to a plethora of pathological changes that suggest significant restructuring of the still developing CNS is to be expected (Lema Tomé et al. 2006b; Kaindl et al. 2008).

Using exclusively or primarily immunohistochemical approaches, our findings in this current study and elsewhere confirm the progressive nature of these changes (Turner et al. 2002, 2007, a, b, c; Lema Tomé et al. 2006a, b, 2007, 2008; Lyall et al. 2009). Indeed, studies that employ standard IHC to define receptor blockade-induced changes in the spatial and temporal distribution of specific gene products will greatly compliment research that uses more cutting edge approaches to target changes in hundreds of such products simultaneously (Kaindl et al. 2008; Turner 2009).

Although the above studies suggest that the developing rodent brain is quite susceptible to NMDAR antagonists, the clinical relevance of this research has often been

questioned. However, a recent study suggests that neonatal exposure to general anesthetics (which act in part or in whole by blocking the NMDAR) can cause cognitive deficits as children mature (Wilder et al. 2009). For the present, this study definitively identified only a narrow range of general anesthetics that likely caused injury, and the full extent of any long-term intellectual impairment is still somewhat speculative. However, the data we have shown here (together with those provided by other groups) suggest both overt as well as subtle cognitive changes may be expected in humans when more exhaustive clinical studies have been completed.

Acknowledgments This work was supported by NIH RO1 NS051632. Injections, perfusions and histology by CPT and CL; image capture, processing and quantification by all authors (except CPT); figure construction by all authors; manuscript preparation by CPT.

References

Abekawa T, Ito K, Nakagawa S, Koyama T (2007) Prenatal exposure to an NMDA receptor antagonist, MK-801, reduces density of parvalbumin-immunoreactive GABAergic neurons in the medial prefrontal cortex and enhances phencyclidine-induced hyperlocomotion but not behavioral sensitization to methamphetamine in postpubertal rats. Psychopharmacology (Berl) 192:303–316

Beals JK, Carter LB, Jevtovic-Todorovic V (2003) Neurotoxicity of nitrous oxide and ketamine is more severe in aged than in young rat brain. Ann N Y Acad Sci 993:115 (discussion 123–114)

Beasley CL, Zhang ZJ, Patten I, Reynolds GP (2002) Selective deficits in prefrontal cortical GABAergic neurons in schizophrenia defined by the presence of calcium-binding proteins. Biol Psychiatry 52:708–715

Bercker S, Bert B, Bittigau P, Felderhoff-Muser U, Buhrer C, Ikonomidou C, Weise M, Kaisers UX, Kerner T (2009) Neurodegeneration in newborn rats following propofol and sevoflurane anesthesia. Neurotox Res 16:140–147

Carlsson A, Hansson LO, Waters N, Carlsson ML (1999) A glutamatergic deficiency model of schizophrenia. Br J Psychiatry Suppl 37:2–6

Dzietko M, Felderhoff-Mueser U, Sifringer M, Krutz B, Bittigau P, Thor F, Heumann R, Buhrer C, Ikonomidou C, Hansen HH (2004) Erythropoietin protects the developing brain against *N*-methyl-D-aspartate receptor antagonist neurotoxicity. Neurobiol Dis 15:177–187

Eastwood SL (2004) The synaptic pathology of schizophrenia: is aberrant neurodevelopment and plasticity to blame? Int Rev Neurobiol 59:47–72

Fredriksson A, Ponten E, Gordh T, Eriksson P (2007) Neonatal exposure to a combination of *N*-methyl-D-aspartate and gamma-aminobutyric acid type A receptor anesthetic agents potentiates apoptotic neurodegeneration and persistent behavioral deficits. Anesthesiology 107:427–436

Golshani P, Truong H, Jones EG (1997) Developmental expression of GABA(A) receptor subunit and GAD genes in mouse somatosensory barrel cortex. J Comp Neurol 383:199–219

Greif KF, Erlander MG, Tillakaratne NJ, Tobin AJ (1991) Postnatal expression of glutamate decarboxylases in developing rat cerebellum. Neurochem Res 16:235–242

Greif KF, Tillakaratne NJ, Erlander MG, Feldblum S, Tobin AJ (1992) Transient increase in expression of a glutamate decarboxylase (GAD) mRNA during the postnatal development of the rat striatum. Dev Biol 153:158–164

Guo Y, Kaplan IV, Cooper NG, Mower GD (1997) Expression of two forms of glutamic acid decarboxylase (GAD67 and GAD65) during postnatal development of the cat visual cortex. Brain Res Dev Brain Res 103:127–141

Harris LW, Sharp T, Gartlon J, Jones DN, Harrison PJ (2003) Long-term behavioural, molecular and morphological effects of neonatal NMDA receptor antagonism. Eur J Neurosci 18:1706–1710

Hashimoto T, Volk DW, Eggan SM, Mirnics K, Pierri JN, Sun Z, Sampson AR, Lewis DA (2003) Gene expression deficits in a subclass of GABA neurons in the prefrontal cortex of subjects with schizophrenia. J Neurosci 23:6315–6326

Hashimoto T, Arion D, Unger T, Maldonado-Aviles JG, Morris HM, Volk DW, Mirnics K, Lewis DA (2008) Alterations in GABA-related transcriptome in the dorsolateral prefrontal cortex of subjects with schizophrenia. Mol Psychiatry 13:147–161

Ikonomidou C, Bosch F, Miksa M, Bittigau P, Vockler J, Dikranian K, Tenkova TI, Stefovska V, Turski L, Olney JW (1999) Blockade of NMDA receptors and apoptotic neurodegeneration in the developing brain. Science 283:70–74

Ikonomidou C, Scheer I, Wilhelm T, Juengling FD, Titze K, Stover B, Lehmkuhl U, Koch S, Kassubek J (2007) Brain morphology alterations in the basal ganglia and the hypothalamus following prenatal exposure to antiepileptic drugs. Eur J Paediatr Neurol 11:297–301

Jevtovic-Todorovic V, Benshoff N, Olney JW (2000) Ketamine potentiates cerebrocortical damage-induced by the common anaesthetic agent nitrous oxide in adult rats. Br J Pharmacol 130:1692–1698

Jevtovic-Todorovic V, Hartman RE, Izumi Y, Benshoff ND, Dikranian K, Zorumski CF, Olney JW, Wozniak DF (2003) Early exposure to common anesthetic agents causes widespread neurodegeneration in the developing rat brain and persistent learning deficits. J Neurosci 23:876–882

Jiao Y, Zhang C, Yanagawa Y, Sun QQ (2006) Major effects of sensory experiences on the neocortical inhibitory circuits. J Neurosci 26:8691–8701

Kaindl AM, Koppelstaetter A, Nebrich G, Stuwe J, Sifringer M, Zabel C, Klose J, Ikonomidou C (2008) Brief alteration of NMDA or GABAA receptor-mediated neurotransmission has long-term effects on the developing cerebral cortex. Mol Cell Proteomics 7:2293–2310

Kristiansen LV, Huerta I, Beneyto M, Meador-Woodruff JH (2007) NMDA receptors and schizophrenia. Curr Opin Pharmacol 7(1):48–55

Lema Tomé CM, Bauer C, Nottingham C, Smith C, Blackstone K, Brown L, Hlavaty C, Nelson C, Daker R, Sola R, Miller R, Bryan R, Turner CP (2006a) MK801-induced caspase-3 in the postnatal brain: inverse relationship with calcium-binding proteins. Neuroscience 141:1351–1363

Lema Tomé CM, Nottingham CU, Smith CM, Beauchamp AS, Leung PW, Turner CP (2006b) Neonatal exposure to MK801 induces structural reorganization of the central nervous system. Neuroreport 17:779–783

Lema Tomé CM, Miller R, Bauer C, Nottingham C, Smith C, Blackstone K, Brown L, Bryan R, Leigh A, Brady M, Busch J, Turner CP (2007) Decline in age-dependent, MK801-induced injury coincides with developmental switch in parvalbumin expression: cingulate and retrosplenial cortex. Dev Psychobiol 49:606–618

Lema Tomé CM, Miller R, Bauer C, Smith C, Blackstone K, Leigh A, Busch J, Turner CP (2008) Decline in age-dependent, MK801-induced injury coincides with developmental switch in parvalbumin expression: somatosensory and motor cortex. Dev Psychobiol 50:665–679

Lewis DA, Levitt P (2002) Schizophrenia as a disorder of neurodevelopment. Annu Rev Neurosci 25:409–432

Lewis DA, Moghaddam B (2006) Cognitive dysfunction in schizophrenia: convergence of gamma-aminobutyric acid and glutamate alterations. Arch Neurol 63:1372–1376

Lu LX, Yon JH, Carter LB, Jevtovic-Todorovic V (2006) General anesthesia activates BDNF-dependent neuroapoptosis in the developing rat brain. Apoptosis 11:1603–1615

Lunardi N, Ori C, Erisir A, Jevtovic-Todorovic V (2009) General anesthesia causes long-lasting disturbances in the ultrastructural properties of developing synapses in young rats. Neurotox Res

Lyall A, Swanson J, Liu C, Blumenthal TD, Turner CP (2009) Neonatal exposure to MK801 promotes prepulse-induced delay in startle response time in adult rats. Exp Brain Res 197:215–222

Mitrovic N, Mohajeri H, Schachner M (1996) Effects of NMDA receptor blockade in the developing rat somatosensory cortex on the expression of the glia-derived extracellular matrix glycoprotein tenascin-C. Eur J Neurosci 8:1793–1802

Olney JW, Farber NB (1995) Glutamate receptor dysfunction and schizophrenia. Arch Gen Psychiatry 52:998–1007

Paxinos G (2004) The rat nervous system. Elsevier, San Diego

Penschuck S, Giorgetta O, Fritschy JM (1999) Neuronal activity influences the growth of barrels in developing rat primary somatosensory cortex without affecting the expression pattern of four major GABAA receptor alpha subunits. Brain Res Dev Brain Res 112:117–127

Remschmidt H (2002) Early-onset schizophrenia as a progressive-deteriorating developmental disorder: evidence from child psychiatry. J Neural Transm 109:101–117

Rice D, Barone S Jr (2000) Critical periods of vulnerability for the developing nervous system: evidence from humans and animal models. Environ Health Perspect 108(suppl 3):511–533

Ringler SL, Aye J, Byrne E, Anderson M, Turner CP (2008) Effects of disrupting calcium homeostasis on neuronal maturation: early inhibition and later recovery. Cell Mol Neurobiol 28:389–409

Slikker W Jr, Zou X, Hotchkiss CE, Divine RL, Sadovova N, Twaddle NC, Doerge DR, Scallet AC, Patterson TA, Hanig JP, Paule MG, Wang C (2007) Ketamine-induced neuronal cell death in the perinatal rhesus monkey. Toxicol Sci 98(1):145–158

Smith C, Lema Tomé CM, Suther E, Lyall A, Ravindra A, Walberg C, Lama SM, Turner CP (2007) NMDAR Blockade: mechansims of age-dependent injury. In: Annual Society for Neuroscience Meeting. 33:243.7, San Diego

Stefovska VG, Uckermann O, Czuczwar M, Smitka M, Czuczwar P, Kis J, Kaindl AM, Turski L, Turski WA, Ikonomidou C (2008) Sedative and anticonvulsant drugs suppress postnatal neurogenesis. Ann Neurol 64:434–445

Turner CP (2009) Perfect storm in a baby's brain. In: Annual Society for Neurosscience Meeting. 39:413.10, Chicago

Turner CP, Pulciani D, Rivkees SA (2002) Reduction in intracellular calcium levels induces injury in developing neurons. Exp Neurol 178:21–32

Turner CP, Miller R, Smith C, Brown L, Blackstone K, Dunham SR, Strehlow R, Manfredi M, Slocum P, Iverson K, West M, Ringler SL, Berry ZC (2007) Widespread neonatal brain damage following calcium channel blockade. Dev Neurosci 29:213–231

Turner CP, Debenedetto D, Liu C (2009a) NMDAR blockade-induced neonatal brain injury: reversal by the calcium channel agonist BayK 8644. Neurosci Lett 450:292–295

Turner CP, Debenedetto D, Walburg C, Ware E, Lambert A, Lee A, Swanson J, Stowe R, Lyle M, Desai P, Johnson R, Liu C (2009b) MK801-induced activated caspase-3 exhibits selective co-localization with GAD67. Neurosci Lett 462:152–156

Turner CP, Ware E, Stowe R, Debenedetto D, Walberg C, Lee A, Swanson J, Desai P, Lyle M, Lambert A, Liu C (2009c) Postnatal expression of GAD67 expression. Neurochem Res. doi:10.1007/s11064-009-0049-y

Waite PME (2004) Trigeminal sensory system. In: Paxinos G (ed) The rat nervous system. Elsevier, Boston, pp 817–851

Wang CZ, Johnson KM (2007) The role of caspase-3 activation in phencyclidine-induced neuronal death in postnatal rats. Neuropsychopharmacology 32:1178–1194

Wang C, Showalter VM, Hillman GR, Johnson KM (1999) Chronic phencyclidine increases NMDA receptor NR1 subunit mRNA in rat forebrain. J Neurosci Res 55:762–769

Wang C, McInnis J, Ross-Sanchez M, Shinnick-Gallagher P, Wiley JL, Johnson KM (2001) Long-term behavioral and neurodegenerative effects of perinatal phencyclidine administration: implications for schizophrenia. Neuroscience 107:535–550

Wang C, McInnis J, West JB, Bao J, Anastasio N, Guidry JA, Ye Y, Salvemini D, Johnson KM (2003) Blockade of phencyclidine-induced cortical apoptosis and deficits in prepulse inhibition by M40403, a superoxide dismutase mimetic. J Pharmacol Exp Ther 304:266–271

Wang C, Anastasio N, Popov V, Leday A, Johnson KM (2004) Blockade of *N*-methyl-D-aspartate receptors by phencyclidine causes the loss of corticostriatal neurons. Neuroscience 125:473–483

Wang CZ, Yang SF, Xia Y, Johnson KM (2007) Postnatal phencyclidine administration selectively reduces adult cortical parvalbumin-containing interneurons. Neuropsychopharmacology 33(10):2442–2455

Wilder RT, Flick RP, Sprung J, Katusic SK, Barbaresi WJ, Mickelson C, Gleich SJ, Schroeder DR, Weaver AL, Warner DO (2009) Early exposure to anesthesia and learning disabilities in a population-based birth cohort. Anesthesiology 110:796–804

Yin HS, Tan HW (2007) Effects of amphetamine on serotoninergic and GABAergic expression of developing brain. Neurotoxicol Teratol 29:264–272