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NMDAR blockade-induced neonatal brain injury: Reversal by the calcium channel agonist BayK 8644

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Abstract

We have previously shown that P7 rat pups injected with the *N*-methyl-D-aspartate receptor (NMDAR) blocker MK801 displayed robust apoptotic injury within hours after injection. Further studies from our lab suggest that loss of calcium cannot be compensated for when vulnerable neurons lack calcium buffering capabilities. Thus, to elevate calcium in these neurons prior to MK801 exposure, we injected P7 rats with the calcium channel agonist BayK 8644. Whereas BayK 8644 did not induce apoptosis by itself, it was found to block MK801-induced injury in a dose-dependent manner. Reversal of MK801 toxicity was complete in the caudate-putamen, partial in the somatosensory cortex but was not observed in the retrosplenial cortex. These results suggest that postnatal brain injury resulting from agents that block the NMDAR, which include commonly used anesthetics as well as drugs of abuse, may be prevented in vulnerable neurons by compensatory increases in calcium prior to exposure to these antagonists.

Keywords

Rat; Postnatal; Apoptosis; Set point hypothesis; MK801; Dizocilpine

We and others have previously shown that blockade of the *N*-methyl-D-aspartate receptor (NMDAR) in postnatal day 7 (P7) rat pups causes profound apoptotic injury [2,12]. In contrast, at P21, agents that block this receptor no longer promote cell death. Numerous mechanisms have been proposed for this age-dependent injury such as loss of neuronal calcium [10–12], disruption of synaptic circuitry, up-regulation of NMDAR protein or absence of trophic support [1,8,9,13,14].

We proposed some time ago that at P7 the regulatory mechanisms that determine optimal calcium levels may not have matured in some neuronal populations [12]. In its simplest form, this calcium set point (CSP) hypothesis [3] states that excessive gain or loss of calcium will result in neuronal injury. In the case of NMDAR blockade, an inability to adapt to loss of calcium entry pushes vulnerable neurons towards cell death [3,10–12]. Indeed, we were the first to show that MK801-induced expression of the pro-apoptotic marker activated caspase-3 (AC3) in P7 rat brains was almost exclusively found in cells that lacked the calcium binding proteins calbindin (CB), calretinin (CR), or parvalbumin (PV) [5], and that gain of these proteins (in particular PV) coincided with loss of MK801 sensitivity [6,7].

This pivotal discovery built on early work from our lab demonstrating that a number of agents that blocked calcium entry or disrupted calcium homeostasis could induce neuronal injury both *in vitro* and *in vivo* [10–12]. Based on these findings, a logical next step would be to counter the loss of neuronal calcium by stimulating entry of this cation through alternative routes just prior to exposure to neonatally toxic agents such as MK801. Thus, we hypothesized that raising neuronal calcium using the calcium channel agonist BayK 8644 would offset the loss of calcium following MK801 blockade of the NMDAR, thereby preventing subsequent neuronal injury. Indeed, we found that MK801-induced apoptosis in P7 animals could be completely blocked by BayK 8644 in the caudate–putamen and partially blocked in the somatosensory cortex. In contrast, BayK 8644 did not influence injury in the ret-rosplenial cortex. These results are discussed with respect to both previous work and clinical implications.

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

Previous work has shown that 1mg/kgMK801 (dizocilpine) can induce profound apoptosis (activation of caspase-3 or DNA damage) in the CNS of P7 rats [2,11,12]. We have also shown that blocking calcium entry through voltage operated calcium channels can promote similar injury [11], suggesting that enhancing calcium entry prior to NMDAR blockade might reduce or prevent apoptosis. Thus, we subcutaneously injected MK801 alone (1 mg/kg; *N*= 6) or together with the calcium channel agonist BayK 8644 (10, 25 or 50 mg/kg; *N*= 4 for each dose; BayK 8644 was injected 15 min prior to MK801). As controls, some P7 animals were injected with BayK 8644 alone (50 mg/kg; *N*= 3) or vehicle (sterile PBS; *N*= 4). Three litters in total were used. After 8 h (a time we have previously determined to be the peak induction of AC3 [11]), animals were anesthetized with 2% isoflurane and perfused with PBS followed by 4% paraformaldehyde in PBS (4% PFA). Brains were then dissected and further fixed in 4% PFA for another 24 h. Tissue was then equilibrated in 10, 20, and 30% sucrose-PBS in preparation for immunohistochemistry.

After 3–4 days in various sucrose solutions (see above), brains were cut frozen into 60µm sections using a sliding (freezing) microtome. We collected coronal sections starting just anterior to the genu of the corpus callosum and stopped collecting at the level of the superior colliculus. Sections were stored in 24-well plates containing PBS such that each row (6wells total) contained serially cut sections. Thus, sections in each well were 6×60µm apart from the preceding section in that well. This was repeated for all brains, and sections from the first well of each row were used to determine levels of AC3 for each animal.

Sections were first incubated in 10mM sodium citrate (pH 6.0) at 95°C for 3–5 min. After rapid cooling, sections were washed with PBS and primary antibody added (rabbit polyclonal anti-AC3, 1:1000; Cell Signaling, Beverly, MA) in IHC buffer (1% BSA, 0.1% TX100, PBS; pH 7.4). After overnight incubation at 4°C, sections were washed in PBS (3 times) and exposed to AlexaFluor-488, goat-anti-rabbit secondary antibody (1:200 in IHC buffer; Invitrogen, Eugene, OR) for 2 h. Sections were then washed in PBS (3 times), mounted onto glass slides (Super frost Plus; Fisher, Pittsburgh, PA), air-dried and then coverslipped using VectorShield mounting media (Vector Labs, Burlingame, CA). Assay controls were performed by omitting the primary antibody, which has been shown elsewhere to abolish immunoreactivity [11,12].

Under UV light, images were captured at 10× or 40× magnification 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 figure display (see Fig. 1) or imported into ImagePro 5.0 (Media Cybernetics, Baltimore, MD) for semi-quantitative analysis (see Fig.

2). As previously described [5], we counted the number of AC3-positive cells within the retrosplenial, somatosensory and caudate–putamen regions (using 10× magnification images). Because these counts were not performed stereologically we used very conservative parameters to estimate the number of AC3 cells in a given brain region [5]. Briefly, within the image frame (1.35 mm × 1.05 mm), we set the size and density thresholds to exclude background or artifact labeling (those cells labeled with arrows were typical of those included in the count; background was established by sampling a zone that was free from specific AC3-immunoreactivity). Size and intensity thresholds were set to exclude background or artifact labeling (size—anything less than 5µm or greater than 100µm; intensity—a value of less than 50 (twice background value), using a scale of 0 = no signal, 255 = highest intensity). Counting was performed automatically by ImagePro 5.0 once these parameters had been set (thus reducing bias).

For the retrosplenial cortex, we counted within the RSGB region (midway from the cortical surface down to the corpus callosum and across to the start of the subcortical white matter) and all layers (I–VI) were counted. For the somatosensory cortex we counted from anterior sections only (SSC_{ANT}; from the level of the tenia tecta to the level of the decussation of the anterior commissure) and included only layers IV and V. For the caudate–putamen (CP), we sampled from four frames that covered the dorsomedial, dorsolateral, ventrolateral and ventromedial regions (these were adjacent but non-overlapping). Sampling from the CP started at the level of approximately the tenia tecta and was terminated just after the emergence of the decussation of the anterior commissure. For the RSGB, we sampled from 6 to 8 sections per animal, for the SSC_{ANT} from 10 to 12 sections per animal and for the CP 8–10 sections per animal.

Mean±SEAC3 cells per region was estimated for each treatment group and differences between the groups determined by a one-way ANOVA, using a Bonferroni post-test comparison of means.

Following injection of P7 rats with BayK 8644 (50 mg/kg), MK801 (1mg/kg) or MK801 (1mg/kg) plus 10, 25 or 50mg/kg BayK 8644 (BM10, BM25, BM50, respectively), brains were processed for AC3 expression 8 h later, as described. In the RSGB, animals treated with BayK 8644 only (BK) displayed little evidence of AC3 (Fig. 1A) but following exposure to MK801 (MK) the number of AC3-positive profiles substantially increased (Fig. 1B). However, even the highest dose of BayK 8644 (BM50) failed to influence MK801-induction of AC3 (Fig. 1C). Semi-quantitative analysis revealed that although there was a mild reduction in AC3 numbers in the BM25 and BM50 groups compared to the MK group, these changes were not statistically significant (Fig. 2A).

In the SSC_{ANT}, the BK group displayed only low levels of AC3 (Fig. 1D) but following MK801 exposure we observed a robust increase in expression of the marker of apoptosis (Fig. 1E). In contrast to the RSGB, we now found that pre-treatment with BayK 8644 could reduce injury in this region (Fig. 1F). Semi-quantitative analysis revealed that, compared to the MK group, although no change in AC3 expression was observed in the BM10 brains, a modest and statistically significant reduction in AC3-positive profiles was observed in the BM25 and BM50 treatment groups (Fig. 2B). The means of both the BM25 and BM50 groups were very similar, suggesting BayK 8644 action had reached its maximum.

In the CP, as with the RSGB and SSC_{ANT}, the BK group displayed only a low level of AC3 immunoreactivity (Fig. 1G). Likewise, compared to other regions, MK801 exposure produced a very robust elevation in expression of the apoptotic marker (Fig. 1H). However, in the presence of BayK 8644, we found a dramatic reduction in AC3-positive cells (Fig. 1I). Semi-quantitative analysis showed that not only were all three dosage groups effective at reducing MK801-induced AC3 expression but that in the BM25 and BM50 groups, the mean AC3 cell

count was now at the same level as that found for the BK group (Fig. 2C), suggesting complete blockade of MK801 toxicity.

We also compared the means of the BK group with those of the vehicle group in all three brain regions. We found that the mean AC3 levels in the BK group was not significantly different from those found in vehicle group in the RSGb (BK group = 13.7 ± 1.5 , vehicle group = 12.2 ± 2.8), in the SSCANT (BK group = 4.9 ± 0.7 , vehicle group = 5.3 ± 1.2) or in the CP (BK group = 9.3 ± 0.9 , vehicle group = 7.4 ± 0.5), as determined by ANOVA (Bonferroni post-test comparison of the means). Further, in the BK group, we found no qualitative evidence for AC3 induction in other brain regions previously studied by this lab (cingulate cortex, motor cortex, thalamus, subiculum, tenia tecta; data not shown). Thus, we feel we can conclude that BayK 8644, by itself, does not induce apoptosis.

We have previously argued that the age-dependent injury resulting from blockade of the NMDAR by MK801 was the result of loss of neuronal calcium [11,12]. In support of this potential mechanism, exposure of rats to the calcium channel antagonist nimodipine promoted similar age-dependent injury [11]. Thus, in this study we asked if raising intracellular calcium by activating calcium channels with BayK 8644 just prior to blocking the NMDAR could reduce the apoptotic injury normally observed following MK801 exposure. Our main finding was a profound loss of AC3-positive cells in the CP region, indicating that transitory elevation of neuronal calcium can prevent injury due to blockade of calcium entry through the NMDAR.

We also studied other brain regions and found BayK 8644 could reduce MK801-induced AC3 expression in the SSCANT, though to a much lesser extent than that found in the CP. In contrast, in the RSGb, we found no evidence in support of BayK 8644's ability to rescue cells destined to die following MK801 injection. Collectively, these data suggest that at the cortical level, raising intracellular calcium to offset loss of this cation following blockade of NMDARs may have only a limited effect on subsequent injury. However, at the sub-cortical level, the same strategy appears to profoundly influence MK801-induced apoptosis. Although these observations are representative of only a few brain regions, they offer the strongest *in vivo* evidence to date that maturing neurons that have not yet acquired the ability to optimally regulate intracellular calcium are vulnerable to agents that prevent calcium entry.

At present we are uncertain why there were regionally-specific outcomes. Differences in pharmacodynamic parameters (regional blood flow, tissue distribution, metabolism, clearance) could explain these observations. However, perhaps the different sensitivities reflect region-specific expression levels of BayK 8644-sensitive calcium channels. Further, these levels may be dynamically regulated by ongoing developmental processes. Alternatively, specific age-dependent expression patterns of multiple proteins (NMDAR, VOCCs, and/or associated proteins) or downstream elements in any given region may be required for BayK 8644 to prevent MK801 toxicity.

Recently, in support of our previous work with CaBPs and the CSP hypothesis, it has been shown that PCP (phencyclidine) also induces AC3 in CaBP-deficient cells [15] and that BayK 8644 can prevent *in vitro* damage caused by PCP [4]. These findings directly support our previous observations which show that the vulnerability of a given population of still developing neurons is linked to the maturity of their calcium buffering capabilities [5–7,10–12]. More importantly, these new developments [4,15] (in combination with our previous discoveries) suggest that the CSP hypothesis can be used as a conceptual platform from which to more fully understand age-dependent injury following NMDAR blockade.

The observations described here and elsewhere [2,11,12] suggest that agents which block the NMDAR and are used in clinical or drug abuse situations may trigger apoptosis in the developing human nervous system. We have shown that one strategy for preventing such injury

could be to elevate intracellular calcium just prior to exposure to these agents. Whereas the opportunity to use this approach may not present itself in drug abuse situations, the strategy would be feasible in clinical situations. For example, prior to exposure to ketamine (a frequently used anesthetic in young children) patients could be injected with an agent that transiently raises neuronal calcium levels. Clearly, application of the CSP hypothesis to this health risk problem may be a fruitful direction to take when developing ways to prevent drug-induced brain injury in young children.

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Fig. 1.

Activated caspase-3 (AC3) expression following MK801 exposure. P7 rat pups were exposed to BayK 8644 (50 mg/kg; BK), MK801 (1mg/kg; MK) or a combination of BayK 8644 (50 mg/kg) plus MK801 (1mg/kg) (BM50). Expression of AC3 in (A–C) the retrosplenial cortex (RSGb), (D–F) the anterior somatosensory cortex (SSC_{ANT}), and (G–I) the caudate–putamen (CP; dorsomedial area shown). Arrows indicate AC3-positive cells; scale in “I” is 100μm. Insets show higher magnification of region outlined in “E” and “F”.

Fig. 2.

Semi-quantitative analysis of AC3 expression. Images were captured (at 10×) and within each 10× frame, the mean number (±SE) of AC3-positive cells was estimated for each treatment group in (A) the retrosplenial cortex (RSGb), (B) the anterior somatosensory cortex (SSC_{ANT}), and (C) the caudate-putamen (CP). Differences in the means determined by ANOVA, using a Bonferroni post-test comparison of the means (** $p < 0.01$, *** $p < 0.001$; NS: not significant).