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MK801-induced activated caspase-3 exhibits selective co-localization with GAD67

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Abstract

Blockade of the *N*-methyl-D-aspartate receptor (NMDAR) in postnatal day 7 (P7) rats can promote rapid and robust induction of the pro-apoptotic marker activated caspase-3 (AC3) and loss of the GABAergic marker GAD67 at P56. Thus, we hypothesized that NMDAR blockade-induced AC3 occurs in GAD67 positive cells at P7. To test this idea, we injected P7 rat pups with vehicle or MK801 and after 8 hours (peak of AC3 induction) we examined brain sections for both AC3 and GAD67. Compared to vehicle, MK801 profoundly induced AC3 in all brain regions examined but co-expression of GAD67 in the same cells was not observed. However, in brain regions where punctate (synaptic) GAD67 was abundant (for example, layer IV of the somatosensory cortex), AC3 was robust. These data suggest that whereas somatic expression of AC3 and GAD67 may be non-overlapping, areas that exhibit punctate GAD67 (and are high in synaptic turnover) may be more vulnerable to MK801 exposure.

Keywords

rat; postnatal; apoptosis; MK801; dizocilpine; synapse; calcium

Introduction

Agents that block the *N*-methyl-D-aspartate receptor (NMDAR) can promote apoptotic injury in the brains of postnatal day 7 (P7) rodents [2,7,8,15,16,18,19]. Recent findings have shown that as animals mature there is loss of a sub-population of neurons expressing both the calcium binding protein (CaBP) parvalbumin (PV) and the γ -amino butyric acid (GABA)-ergic marker, glutamic acid decarboxylase 67 (GAD67) [1,20]. This would suggest that cells which are phenotypically PV, GAD67, or both are at risk to NMDAR blockade at P7 and that at least a sub-population of GAD67 neurons should display evidence of apoptosis at this earlier age.

We have previously shown that expression of AC3 following MK801 exposure does not occur in CaBP-expressing neurons, including PV [10]. We therefore asked whether NMDAR blockade-induced AC3 occurred in GAD67-positive cells. Thus, P7 rats were exposed to vehicle or MK801 and, after 8 hours, immunohistochemically stained for the pro-apoptotic and

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GABA-ergic markers. Whereas expression of AC3 and GAD67 did not occur in the same cell bodies, we did observe robust AC3 expression in areas displaying GAD67-rich puncta. The implications of these results are discussed with respect to drug-induced injury and postnatal development.

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, perfusions

Earlier studies have shown that 1 mg/kg MK801 (dizocilpine) can promote rapid and robust expression of the apoptotic marker AC3 in P7 rat brains [7,15,16]. Thus, we subcutaneously injected P7 rat pups with vehicle (sterile phosphate buffered saline (PBS); pH 7.4; N = 5) or MK801 (1 mg/kg in PBS; N = 5). In each treatment group, pups were of mixed gender (of roughly equal numbers). Eight hours after injection (peak time of induction of AC3 [15]) 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 hr.

Immunohistochemistry (IHC)

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 μ m 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 $\times$ 60 μ m apart from the preceding section.

To optimize AC3 staining, sections were first exposed to 10 mM sodium citrate, pH 6.0, at 95°C for 3–5 min [15]. After rapid cooling, sections were washed with PBS and primary antibody was 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 hr. After washing in PBS (3 times), sections were then incubated with a mouse anti-GAD67 primary antibody (1:400 in IHC buffer; Millipore, Temecula, CA) for 24 hr, washed in PBS, and then exposed to an AlexaFluor-594, donkey, anti-mouse secondary antibody (1:200; Invitrogen) for 2 hr. 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). In both the AC3 and the GAD67 steps, primary antibody omission served as an assay control.

Imaging and Quantification

Under UV light, images were captured at 10X, 20X, 40X or 60X 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).

Activated caspase-3 counts—We counted the number of AC3-positive cells within the cingulate, somatosensory and caudate-putamen regions. Because these counts were not performed stereologically we used very conservative parameters to estimate the number of

AC3 cells in a given brain region (see Lema Tomé et al for details [10,11]). Cell counts were expressed as mean density per section for each region, across 6–8 sections per animal. Counts performed by all authors except CL, CPT and RJ.

For the cingulate cortex, we only counted within the Cg2 region as this was where the majority of AC3-positive cells were found for this region. For the somatosensory cortex, we counted from anterior sections only (SSC_{ANT}). For the caudate-putamen (CP), we sampled from four 10X frames that covered the dorsomedial, dorsolateral, ventrolateral, and ventromedial regions (which were adjacent but non-overlapping). Areas were defined according to Paxinos [14].

Western Blot Analysis: P7 rat pups were subcutaneously injected with vehicle (N = 5) or MK801 (1 mg/kg; N = 5), anesthetized (2% isoflurane) after 8 hr, and brains quickly removed. On ice, each brain was micro-dissected into various brain regions and tissue was placed into microfuge tubes, frozen on dry ice and stored at –80°C until needed. Following protein extraction, a small volume of each sample (each containing 25 µg of protein) was mixed with loading buffer and loaded into a single well of a polyacrylamide gel (12%). Separated proteins were transferred to nitrocellulose membranes (Trans Blot Transfer Medium, BioRad, Hercules, CA), which were then probed for GAD67 expression (mouse anti-GAD67, 1:5000, Millipore; donkey HRP-conjugated anti-mouse secondary, 1:5000, Jackson Immuno Research, West Grove, PA) or AC3 expression (rabbit anti-AC3, 1:1000, Cell Signaling; goat HRP-conjugated anti-rabbit secondary, 1:5000, Amersham, UK). Membranes were stripped and probed for β-actin (as an internal control; mouse-anti-β-actin, 1:5000, Sigma, St Louis, MO; HRP-conjugated anti-mouse, 1:5000, Amersham, UK). Visualization of bands was performed using ECL solution (Amersham, UK) and protein levels were determined based on their band density on exposed film (Biomax XAR, Kodak, Rochester, NY). The density for GAD67 or AC3 was normalized to the corresponding β-actin for that lane and the mean (normalized) band density (± SE) across all animals in a given treatment group determined.

Statistical Analysis: The mean number of AC3 positive cells (± SE) 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. Mean band density for each protein was estimated by Western blot (see above) and differences in the means determined using a two-tailed Students *t*-test. All statistical analyses were performed using GraphPad Prism, version 4.0 (GraphPad, San Diego, CA).

Results

MK801 induces activated caspase-3 (AC3)

P7, P14 or P21 rat pups were injected with vehicle or MK801 (1 mg/kg) and after 8 hr brains were prepared for AC3 staining (see Methods). At P7, in the Cg2 region, there were low levels of AC3 immunoreactivity (-ir) in sections from vehicle-treated animals (Fig. 1A1). However, following MK801 injection, we found there was a robust elevation of this marker of apoptosis (Fig. 1A2). Quantitative analysis revealed that MK801 produced a substantial and highly significant increase in AC3 in the Cg2 that was approximately 6-fold greater than vehicle controls (Fig. 1A3). At P14, MK801 action was far less impressive and AC3 levels were approximately 2-fold higher than that found for vehicle (Fig. 1A3). At P21, there was almost no detectable AC3 in either the vehicle or MK801-treated groups (Fig. 1A3). Similarly, in the SSC_{ANT} (Fig. 1B1–B3) and CP (Fig. 1C1–C3), robust AC3-ir was induced by MK801 at P7 (13-fold in the SSC_{ANT}; 7-fold in the CP). At P14, sections from MK801-treated animals displayed approximately a 2-fold increase over that found in sections from vehicle-treated animals (for both the SSC_{ANT} and CP). At P21, there were very few AC3-positive cells in either treatment group (SSC_{ANT} and CP). Although previous work from this lab collectively

shows an age-dependent decline in MK801-induced cell death [10,11,15,16], Fig. 1 presents these data for the first time as a continuous set in the same study. Further, these data allow the rest of this current study to be placed in a broader context. More importantly, Fig. 1 emphasizes that P7 is the critical age at which we should examine how MK801-induced-AC3 relates to expression of other proteins of interest, for example GAD67.

Co-expression of AC3 and GAD67 in synapses not soma

Sections from the brains of vehicle- or MK801-treated animals were co-stained for both AC3 and GAD67 as described (see Methods). Under UV light, images were captured using filters selective for 488 nm (green, AC3) or 594 nm (red, GAD67) wavelengths. Because we wished to examine GAD67 as it relates to AC3 induction following MK801 exposure, only sections from MK801-treated, P7 animals are described. However, in agreement with previous reports [3–6,9,22], GAD67 levels were generally low in most brain regions, with somatic expression in particular either absent or present in a region-specific manner (compare all panels in Fig 2).

In the Cg2, AC3 expression was robust in local areas that were also immuno-positive for punctate GAD67 (layer V, for example; Fig. 2A). In the SSC_{ANT}, abundant AC3 was found mostly in layer IV (and less so in layer V), and was associated with punctate GAD67-ir (Fig. 2B). In both cortical regions it was rare to see somatic GAD67-ir. In the CP, local zones rich in GAD67-positive cell bodies and/or fibers appeared to be surrounded by AC3-positive cells (Fig. 2C), a distribution very reminiscent of the mutually exclusive zones we have previously described for MK801-induced AC3 and calbindin-D28K in this same structure [10]. GAD67-positive cell bodies were more numerous in the lateral aspect of the CP but none were positive for AC3 (Fig. 2D).

The one region where GAD67-positive cells were abundant was the thalamic reticular nucleus (TRN). Here, somatic GAD67-ir was particularly robust but AC3 expression was found only at the borders of this structure (Fig. 2E and F). In contrast, in a number of other thalamic nuclei (for example, the anterior dorsolateral nucleus - ADL) exhibited punctate rather than somatic GAD67-ir and in these structures high levels of AC3 expression were observed (Fig. 2G and H). Collectively, these data suggest that induction of apoptosis does not occur in GABAergic soma but instead overlaps with striking regularity wherever we find GABAergic terminals (at least in the brain regions examined here).

No change in GAD67 despite robust induction of AC3

We next asked if MK801 treatment altered overall expression levels of GAD67 in brain regions where we also observed robust induction of apoptosis. We focused on the SSC_{ANT} of P7 animals because we observed high levels of AC3 induction within a GAD67-rich area (layer IV). To quantify changes, we performed Western blot analysis of tissue from the SSC_{ANT} of vehicle or MK801-treated P7 animals 8 hr after injection (see Methods). We found that expression of GAD67 was unaltered following MK801 injection (Fig. 3A). In contrast, parallel samples from the same tissue displayed an approximately 3.5-fold induction of AC3 in the MK801-treated group compared to the vehicle-treated group (Fig. 3B). These data suggest that MK801 had potentially engaged the apoptotic machinery without altering overall GAD67 expression (at least at 8 hr post-injection, a time typically associated with peak induction of AC3 [15]).

Discussion

NMDAR blockade at P7 (or earlier) leads to widespread apoptosis in the CNS [7,15,16] that at P56 (or later) is associated with loss of neurons positive for both the GABAergic marker, GAD67, and the CaBP, PV [1,20]. It might seem reasonable, therefore, that this early apoptotic

event is directly linked to later loss of the GAD67-PV phenotype. If true, we should expect that at P7, MK801-induced AC3 co-localizes to either GAD67 or PV expressing cells. In a previous study, we have found AC3 induction following NMDAR blockade occurs in cells absent for CaBPs, including PV [10]. Thus, in the present study, we focused on the relationship between MK801-induced AC3 and GAD67.

Punctate but not somatic GAD67 is associated with apoptosis

At the age studied here, there were very few cell bodies positive for the GABAergic marker in the cortical or striatal regions examined. Even when GAD67 cells *were* present, co-expression of AC3 and GAD67 in the same cell was not observed (Fig. 2). Indeed, when we expanded our analysis to the TRN, a region rich in somatic GAD67 (Fig. 2), induction of AC3 occurred only at the borders of this thalamic nucleus. In contrast, brain regions displaying GAD67-rich puncta (for example the barrel field zones (layer IV) of the somatosensory cortex) were associated with robust MK801-induced AC3. Given that the whisker input that provides the sensory drive to this region is still rapidly maturing [17,21], synaptic turnover is likely to be high.

It must be stated that our data are not of sufficient resolution to distinguish whether the GAD67-puncta are terminating directly on the AC3-positive cells or are simply adjacent to such cells. Indeed, confocal analysis could be a critical design element in our future studies. Regardless, in the current study, wherever we examined AC3-rich areas at high magnification, GAD67-puncta rather than soma predominated in the same field. Perhaps then, brain regions that are highly plastic are especially vulnerable to NMDAR blockade at P7. If so, why did we observe AC3 induction around but not within GAD67-rich patches in the CP? In these patches there was a mix of fibers and cell bodies as well as puncta. Thus, in any given region, the nature of GAD67-ir (somatic versus terminal) may be one of many factors that determine whether AC3 will or will not be induced.

MK801 injury and GABAergic termini

Levels of GAD67 are generally low at P7 and do not reach adult levels until very late in the postnatal period [3–6,9,22]. In this sense, the P7 age contrasts sharply with the end of the postnatal period: robust AC3 at a time when somatic GAD67 is low, low to absent AC3 when somatic GAD67 is elevated. This is remarkably similar to changes in PV expression at these same ages [12,13], suggesting that the onset of mature expression patterns of both markers takes place at ages associated with a relative absence of MK801 toxicity.

This inverse relationship would seem counter-intuitive to the observation that NMDAR blockade at P7 (or younger) promote loss of the GAD67-PV phenotype at later ages (P56 or older) [1,20]. However, we show that MK801-induced AC3 is robust in areas containing GAD67-rich puncta, implying that many of the cells within the local network that receives these GABAergic terminals are destined to die, subsequently depriving their targets of trophic support. Days, weeks, or several months may pass before secondary pathologies in GAD67 soma are revealed. Alternatively, the disappearance of the GAD67-PV phenotype at later ages [1,20] may be a result of disrupting the normal developmental program of the GAD67-PV subpopulation. On the other hand, AC3 induction may have occurred in cells whose GAD67 gene has yet to be activated and the non-overlapping relationship between AC3 and GAD67 we have described may be more apparent than real. Work is in progress to determine which of these interpretations are mechanistically consistent with events associated with drug-induced injury in the postnatal brain.

Whereas a clear inverse relationship has been demonstrated between MK801-induced AC3 and CaBP expression in the P7 rat brain [10,11], the relationship between AC3 induction and

GAD67 seems more complex, with somatic GAD67 expression displaying mutually exclusive patterns with AC3 and punctate GAD67 expression displaying co-localization with AC3. Regardless, induction of apoptosis in P7 animals may depend on many factors, at least two of which we believe our research to date has now identified: the absence of a CaBP and the presence of GABAergic terminals. We believe that unraveling the mechanisms behind MK801-induced cell death will generate more surprising discoveries before the bigger picture emerges.

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Fig. 1. MK801 induces activated caspase-3 (AC3) expression

Following exposure to vehicle or MK801, brain sections from P7, P14, or P21 pups were processed for AC3 expression by IHC (see Methods). Expression of AC3 in: (A1–A3) the cingulate cortex (Cg2); (B1–B3) the somatosensory cortex (SSC_{ANT}); and (C1–C3) the caudate-putamen (CP). Images show sections from vehicle-treated (A1, B1, C1) or MK801-treated pups (A2, B2, C2). Mean number AC3 positive cells ($\pm$ SE) per treatment group across all ages in Cg2 (A3), SSC_{ANT} (B3) or CP (C3). Roman numerals indicate location of layers; scale bar in C2 is 200 μ m; arrows indicate AC3-positive cells. Statistical differences determined by ANOVA, using a Bonferroni post-test comparison of means (* p <0.05, *** p <0.001).

Fig. 2. Expression patterns of AC3 and GAD67 following NMDAR blockade

Following MK801 exposure, brains sections from P7 animals were processed for both AC3 and GAD67 as described (see Methods). In all panels, arrows indicate AC3-ir, arrowheads GAD67-positive puncta, and asterisks GAD67-positive cell bodies. **(A)** Layer V of the cingulate cortex (Cg2). Please note that AC3-positive cells are surrounded by GAD67-rich puncta. **(B)** Layer IV of the anterior somatosensory cortex (SSC). Please note the two AC3-positive cells that are surrounded by GAD67-rich puncta. **(C)** Ventral area of the caudate-putamen (CP). Please note that AC3-positive cells fall outside zones of high GAD67-ir. **(D)** Lateral area of the caudate-putamen. Please note that somatic AC3 does not overlap with somatic GAD67. **(E)** Low power view of the thalamic reticular nucleus (TRN – boundaries

defined by the dashed lines). Note how AC3-positive cells are located at the border but none are found within the TRN interior. **(F)** High power view of box indicated in E. The TRN has a high density of GAD67-positive cells but almost none appear to be AC3-positive following MK801 exposure. **(G)** Low power view of the thalamic anterior dorsolateral nucleus (ADL). Please note the numerous AC3 profiles in the panel. **(H)** High power view of box shown in G. Please note the how the numerous AC3-positive cells are surrounded by GAD67-rich puncta. Scale in A, B, D, F and H is 50 μm ; scale in C, E and G is 200 μm .

Fig. 3. Western blot analysis for GAD67 and AC3 at P7

P7 rat pups were treated with vehicle (N = 5) or MK801 (N = 5) and 8 hr later brain tissue was processed for GAD67 or AC3 expression by Western blot analysis (see Methods). **(A)** Blot of GAD67 expression in SSC_{ANT} of vehicle (Veh) or MK801 (MK) treated animals. The corresponding β -actin blot is shown below and mean band density of GAD67 signal (normalized to β -actin) shown right. **(B)** Blot of AC3 expression in SSC_{ANT} of vehicle or MK801 treated animals. The corresponding β -actin blot is shown below and mean band density of AC3 signal (normalized to β -actin) shown right. Statistical differences assessed by Student's t-test (***) $p < 0.001$; NS – not significant).