Title
Intrathecal infusion of BMAA induces selective motor neuron damage and astrogliosis in the ventral horn of the spinal cord

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Abstract: The neurotoxin beta-N-methylamino-L-alanine (BMAA) was first identified as a "toxin of interest" in regard to the amyotrophic lateral sclerosis-Parkinsonism Dementia Complex of Guam (ALS/PDC); studies in recent years highlighting widespread environmental sources of BMAA exposure and providing new clues to toxic mechanisms have suggested possible relevance to sporadic ALS as well. However, despite clear evidence of uptake into tissues and a range of toxic effects in cells and animals, an animal model in which BMAA induces a neurodegenerative picture resembling ALS is lacking, possibly in part reflecting limited understanding of critical factors pertaining to its absorption, biodistribution and metabolism. To bypass some of these issues and ensure delivery to a key site of disease pathology, we examined effects of prolonged (30 day) intrathecal infusion in wild type (WT) rats, and rats harboring the familial ALS associated G93A SOD1 mutation, over an age range (80±2 to 110±2 days) during which the G93A rats are developing disease pathology yet remain asymptomatic. The BMAA exposures induced changes that in many ways resembles those seen in the G93A rats, with degenerative changes in ventral horn motor neurons (MNs) with relatively little dorsal horn pathology, marked ventral horn astrogliosis and increased 3-nitrotyrosine labeling in and surrounding MNs, a loss of labeling for the astrocytic glutamate transporter, GLT-1, surrounding MNs, and mild accumulation and aggregation of TDP-43 in the cytosol of some injured and degenerating MNs. Thus, prolonged intrathecal infusion of BMAA can reproduce a picture in spinal cord incorporating many of the pathological hallmarks of diverse forms of human ALS, including substantial restriction of overt pathological changes to the ventral horn, consistent with the possibility that environmental BMAA exposure could be a risk factor and/or contributor to some human disease.

May 16, 2014

Ahmet Hoke
Editor-in-Chief
Experimental Neurology

Re: Ms. No.: EXNR-14-173
Title: Intrathecal infusion of BMAA induces selective motor neuron damage and astrogliosis in the ventral horn of the spinal cord

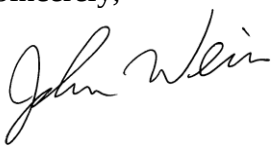
Dear Dr. Hoke:

Thank you for arranging review of our paper, "Intrathecal infusion of BMAA induces selective motor neuron damage and astrogliosis in the ventral horn of the spinal cord".

I am extremely embarrassed by the mistake found by the first reviewer (with a graph depicting the data from Fig. 6 attached to Fig. 2 – instead of the correct graph), and am very grateful for the opportunity to resubmit the paper as a revision and address the reviewers' comments and concerns. I have inserted the correct graph, written a detailed "response to reviews" note, and made a number of additions and clarifications to the manuscript to address the concerns and suggestions of both of the reviewers.

In summary, I thank the reviewers for the careful reading, for finding the error in Fig. 2, and for the numerous questions and requests for clarifications. In trying to address them I feel that the paper is much improved and I hope that you will find it suitable for publication in *Experimental Neurology*.

Sincerely,



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In addition, we request that Drs. Mark Duncan, TJ Montine, Daniel Perl, Patrick McGeer, and Christopher Shaw not be selected as reviewers.

Re: Ms. No.: EXNR-14-173
Title: Intrathecal infusion of BMAA induces selective motor neuron damage and astrogliosis in the ventral horn of the spinal cord

Responses to the reviews:

Reviewer #1:

We are pleased that despite the serious concerns substantially arising from our error in final manuscript preparation, the reviewer finds that "*This is an interesting study of pathologic changes induced by 30-day intrathecal infusions of BMAA into WT and G93A SOD1 mutant rats"*.

In regard to this reviewer's specific comments and concerns:

1. Most concerning is that there is a bar graph in figure 2 that is supposed to show mean motor neuron counts in ventral horns under the various experimental conditions. There is another bar graph in Figure 6, which is supposed to be the "mean fluorescence intensity in the surround regions" of MNs in sections immunostained for GLT-1. The scale on these two bar graphs is the same (0-50) without any units given, which seemed unusual since one is supposed to be cell counts and the other is supposed to be a completely unrelated measure of fluorescence intensity in a separate set of staining experiments. The y-axis in the figure 2 graph is stretched out compared to figure 6, but by copy/pasting the figures into a separate document and scaling the y-axes so that the scales match, it becomes clear that the figures are actually showing identical data. The columns and error bars are exactly the same in these two bar graphs. It is unclear if this is an honest mistake or an example of fraud. Assuming this is an honest mistake, it is important to know which is the correct figure.

Although I was quite upset when I first read this review, and noted that the reviewer was absolutely correct and a graph depicting the wrong data was mistakenly inserted under Fig. 2 (which does indeed reflect the data from Fig. 6, quantifying the GLT-1 intensity surrounding MNs), I truly wish to thank the reviewer for noting the problem at the review stage (…far better than not catching it until after publication)!

I tried to reconstruct how such an error could have occurred. At an earlier stage of draft preparation we did have a version of Fig. 2 with the correct graph attached. The data for each of the figures was contained in excel spread sheets containing all the counts, measurements, statistics and the corresponding graphs. In the late stages of preparation we decided to change the y-axis labels (which were inserted in powerpoint text boxes), and to make the change, the graph was copied from excel into the powerpoint file. Apparently the graph for the data in fig 6 was copied into the new powerpoint layout for fig. 2. I wondered how this could have slipped through the final proofing unnoticed – and all I can think of is that the graphs superficially look fairly similar – with the same 4 conditions, and both with the control (WT+saline) condition showing the greatest value with significant decreases in the other three conditions.

The correct data – now attached to Fig. 2 - does indeed show BMAA and mutant SOD1 both to cause a far greater degree of MN damage (close to 50%) than had be wrongly suggested by the Fig. 6 graph (about 20%) .. and it occurs to us that the relative paucity of damage that had been

previously suggested by this graph could have diminished confidence in the significance and objectivity of the reported MN loss / damage.

Additionally, both figures should ideally be normalized to the control condition and expressed as a percent change. In figure 2, the legend says that the counts were the mean values from 5-9 animals - How many animals were in each condition? The legend says in parentheses that there were 9 animals in the WT+BMAA group, but doesn't give numbers for the other groups.

In Fig 2, the values represent the mean number of intact ventral horn MNs counted per spinal cord section examined (comprising two – bilateral – ventral horns per section). We felt that this number was useful as it gives a measure of the actual numbers of healthy MNs judged to be present and we would prefer to leave it this way. Regarding the data in Fig. 6, the values represent arbitrary fluorescence intensity measures, and agree that it would make more sense normalized to control. We appreciate the suggestion and have done so.

Regarding the numbers of animals examined in each condition, we did indeed have the greatest number of animals compiled for the WT+BMAA condition. The reason we emphasized this number is that the counts in the control (WT+saline) animals were quite consistent (note the error bar is smallest in this condition), the changes in the SOD1 mutant animals have been very well documented by ourselves and others, and we felt that the effect of BMAA constituted the new information about which we wanted to be completely confident. In any event – as indicated, at least 5 animals comprising hundreds of slices and thousands of MNs were counted in each condition. We now indicate the exact number of animals examined in each condition (WT+Saline 6; WT+BMAA 9; Tg+Saline 5; Tg+BMAA 5) in the legend to Fig. 2.

2. While it may be interesting that a 30 day intrathecal infusion of BMAA resulted in pathologic features that could mimic some changes seen in ALS, is it actually possible for environmental exposure to ever produce this high of a level of BMAA in the spinal fluid? How was the intrathecal dosage chosen? How much BMAA would have to be ingested PO to cause this level in the CSF? Why was 30-days chosen as the length of time? The authors mention the primate model of BMAA ingestion and that it is controversial because of "low potency of BMAA" - in fact it has been estimated that a human would have to eat their body weight in cycad flour every single day to approximate the amounts ingested in that study. Since elevated BMAA levels have been found in autopsy brains of ALS patients from wide ranging geographical areas and cyanobacteria producing BMAA are ubiquitous, some researchers have hypothesized that certain individuals may have a genetic susceptibility that results in BMAA accumulation in the brain tissues.

These are all good questions for which there are no precise answers and many unknowns. Regarding the level of the BMAA exposure: We do not know the precise steady state levels arising from our slow intrathecal infusion of concentrated BMAA – but as described in the conclusions section of the paper, we make a rough estimate that a level in the low 10's of micromolar seems likely. Furthermore, such a level is compatible with levels at which we have previously found BMAA to induce slow and relatively selective damage to MNs in dissociated spinal cord cultures. Part of the reason for the exposure level used was empiric – we carried out a prior trial with 2 rather than 5 mM BMAA loaded into the minipumps, with infusions carried out for 2 rather than 4 weeks and saw little MN damage, so our exposure seems likely to be near

the threshold needed to produce clear MN damage over several week exposure.

Regarding levels that may occur in humans, these are also big unknowns, but there are some reasons to believe that substantial exposures, possibly comparable to those used here – might at least on occasion occur. Regarding the statement that one would have to eat ones weight in cycad flour to get the dosage of BMAA used in Peter Spencer's primate study – that was based upon old measures in washed and unhydrolyzed cycad flour. However, a key study reported that acid hydrolysis dramatically increased recovery of BMAA from cycad flower (as well as from cyanobacteria, animals and humans in which it was found, providing the first indication that BMAA appears either to associate closely with or be incorporated into proteins; *Murch et al. 2004a [PNAS 101: 12228-31]*). Of far greater concern regarding possible human exposures, however, are the studies over the past decade reporting BMAA to be produced by diverse taxa of cyanobacteria found worldwide, and the possibility of exposure resulting from human ingestion either of cyanobacteria, or of organisms obtained from aqueous environments where cyanobacterial blooms had occurred. Some studies of marine organisms have reported very high levels of BMAA in acid hydrolyzed tissues from marine animals (see *Brand et al., 2010 [Harmful algae 9: 620-635]; Jonasson et al., 2010 [PNAS 107: 9252-7]*). Suggesting a possible link to human disease, high levels of BMAA have been reported to be present in brain or spinal cord tissue of human sporadic ALS and Alzheimer's disease patients (see *Pablo et al., 2009 [Acta neurologica Scandinavica 120: 216-225]*). In that study, reported BMAA levels in hydrolyzed brain and spinal cord tissues of diseased patients ranged from ~30 – 250 μg/g, which, for a compound with MW ~ 120, free in solution would correspond to concentrations in the hundreds of micromolar to low millimolar range. The hypothesis would be that the BMAA accumulation reflects repeated dietary exposures, with uptake into the CNS (which has been documented to occur after systemic administration and appears to be an important site of long lasting BMAA accumulation and potential incorporation into proteins; see *Xie et al, 2014 [Neuroreport 24: 779-84]*), likely combined with differences in risk between individuals due to genetic variability in factors such as uptake, transport or metabolism. Little is known about potential human exposures, which are the subject of epidemiological studies – yet, if dietary consumption of foods containing levels of BMAA reported in some of the studies occurred, along with CNS uptake as reported, it may be possible that free extracellular levels in the 10's of micromolar could at least transiently occur after consumption. In addition, if as has been suggested, BMAA is incorporated into protein (see *Dunlop et al., 2012 [Plos One 8: e75376]*), large quantities of bound BMAA stores could accumulate, with some level of free BMAA present under steady state resulting from protein turnover. Although BMAA can clearly act as an extracellular excitotoxin (see recent review: *Chiu et al., 2011 [Int J Environ Res Public Health 8: 3728-46]*), it could well have diverse effects mediated through different mechanisms, and it is not known which combinations of effects are most likely to result in chronic pathology.

We have made additions in both the introduction (p. 3-4) and the discussion (p. 15-16) that attempt to more clearly discuss the current understanding and outstanding questions as to possible sources of BMAA and its potential entry into and accumulation in the central nervous system.

3. *Did these animals have a phenotype? Did they become weak? The authors state that they chose the timing of the infusions because they wanted a time when there were early pathological changes apparent in the SOD1 animals. It seems it would have been more interesting to choose a time point and/or length of infusion that might result in a motor neuron disease phenotype. As*

they are claiming that the pathologic changes approximated the SOD1 rats, why not continue the infusions for 60-80 days to see if they become weak like the SOD1 animals?

These animals did not develop a clinical phenotype during the period of the infusion, and if our interest is modeling what might possibly be occurring in humans who are subjected to dietary BMAA exposures, we would likely not expect them to, as there is no evidence for relatively acute BMAA linked illness in humans and any human clinical disease linked to consumption would most likely only be seen after many years and repeated exposures. Our thought in planning the studies was to infuse the BMAA during the evolving presymptomatic stages of SOD1 mutant linked disease in order to provide what we thought was the best opportunity to see clear synergism. As far as the possibility of doing infusions much longer than the 30 days, we have tried that without much success, as the intrathecal catheters often became occluded, apparently due to local inflammatory tissue reactions causing luminal plugging, such that very few remained patent at 60 days.

4. For the data in Figure 2, the authors state that MNs with pyknotic nuclei, marked atrophy, or swelling, or a highly irregular or fragmented soma were not counted as alive. What percentage of the decreased counts were due to these MNs assessed to be abnormal? Was this counting conducted in a blinded fashion? There does not appear to be too much difference between panels A (WT+Saline) and B (WT +BMAA), except that the staining is a little darker in panel B. Also, the lower power pics don't really add anything because you can't see anything on them. If anything, the low resolution pictures make it look like there was MORE SMI-32 staining in the TG+Saline group and substantially more in the TG+BMAA group, but this may be an artifact of darker staining. The lettering of A, B, C, D isn't needed since the panels are separately labeled, and the letters aren't explained in the legend anyway. Additionally, the authors state that there was no synergism between SOD1 G93A mutants and BMAA treatment, but the bar graph in figure 2 possibly suggests at least a trend (assuming this is the real data and not the data for figure 6). Depending on how many animals were used, it may be significant - it appears that different numbers of animals (5-9?) were used in the different groups.

Regarding the MN counts, numbers of MNs in different states varied somewhat from slice to slice – but in general, most of the decreases in MN numbers in conditions other than WT+saline were *not* due to complete disappearance of the MNs but rather to severe pathological changes as described, with examples highlighted by arrows / arrowheads in the images in Fig. 1 and 2. The fields illustrated are just representative ones to illustrate some of the types of changes seen, from hundreds of fields evaluated. The low power images just show the regions of the ventral horns (as well as dorsal horns for Fig. 1) illustrated in the high power images. Regarding differences in the appearance of the MNs in the different conditions, we found almost all MNs in the WT+saline condition to have good morphology, with far more MNs showing either swelling, constriction, fragmentation of processes or microglial infiltration in the other conditions, as indicated by the arrows/arrowheads in both figs 1 and 2. Regarding synergism, in the present study we were wondering if we would see gross synergism, with substantially more injury in the combined exposure that with either BNAA or mutant SOD1 alone, and we clearly did not see that. There could well be some sort of synergism of the effects of BMAA and mutant SOD1, but we do not feel that we can reliably detect it with the present paradigm. Finally, we have removed the unnecessary panel labeling (A, B, C, D) and thank the reviewer for suggesting this!

5. In figure 3 it that the density of motor neurons is actually lowest in the WT+Saline field. While this figure is supposed to be showing the TDP-43 inclusions, there clearly appears to be a higher density of motor neurons in the WT+BMAA and especially in the TG+BMAA panel compared to panels A & C. While this might just be unfortunate selection of fields for these figures, it seems to contradict the conclusions being made by figure 2.

The TDP-43 stain does not provide good visualization of the MN somata, and for this reason all the MNs in the fields are not immediately evident. Most of the cells with the large, nearly round nuclei are MNs, and to our count there are ~ 7-9 MNs contained in each of the photographed fields. MN density does vary a bit from region to region and, as indicated, quantifications of intact surviving cells (for Fig. 2) are based on counts from hundreds of ventral horns. A large part of the reason that the MNs may appear more evident in the non control conditions is that in many of the severely damaged cells, the TDP-43 labeling is not restricted to the nucleus (as it is in the control condition), and this makes the soma more evident. Such cells, indicated by arrows, would often have damaged somata as indicated by Nissl (Fig. 1) or SMI-32 (Fig. 2) labeling, and likely would not have been counted as surviving for the data presented in Fig. 2.

5. Figure 4 suggests astrogliosis may have been more diffuse - with reactive astrocytes increased in dorsal horns as well as ventral horns. Therefore, an alternative hypothesis might be that the BMAA is acting nonspecifically on the astrocytes, and then motor neurons have a selective vulnerability to factors produced by reactive astrocytes. ie, it may not be that BMAA has a selective effect on the motor neurons. If it did, how do you explain the increased gliosis in dorsal horns?

In our astrocyte cell counts, we did not find significant increase in numbers of reactive astrocytes in dorsal horn, either with BMAA or with mutant SOD1. We wonder if the reviewer might have misunderstood the labeling on figure 4; the "#" symbol above the DH bars indicates that the number of astroctyes was less than were present in VH with the same treatment. However, although not significant, we do see a suggestion of an increase in reactive astrocyte numbers in DH in the Tg+BMAA condition, possibly compatible with prior findings that astrogliosis is not completely selective for VH in the SOD1 model (with some diffuse spinal gliosis seen in symptomatic disease); this finding may also suggest some degree of synergism between effects of BMAA and the mutant SOD1. As far as potential mechanisms of astrocyte effects, there are a number of mechanisms in addition to direct excitotoxicity through which BMAA has been suggested to act, and it is certainly possible that BMAA could have some direct effects on astrocytes (as we now state in the manuscript, p. 13).

6. The images in Figure 6 are far from convincing. Higher quality images and better control of the gain (background fluorescence) would be helpful.

We have applied a level adjustment to decrease contribution of background fluorescence and modestly amplify differences between midrange fluorescence values as suggested for all the images in Fig. 6, and feel that this does help to bring out the differences in GLT-1 labeling in the region immediately surrounding the MNs.

Reviewer #2:

We are pleased that the reviewer feels that *"The paper is ACCEPTABLE for publication: it is well designed, carefully executed, well written, and timely".*

In regard to this reviewer's specific comments and concerns:

There are few weaknesses that should be addressed:
- purity of L-BMAA is not stated
- stability of L-BMAA in solution is not addressed
- assumption that L-BMAA and not a metabolite is causing the effects described is not addressed

Regarding the purity and stability of the BMAA, while we do not have precise characterization of these measures, it was commercially prepared and purified, and has been used by us as well as other investigators with consistent and stable results. We have often kept BMAA in solution for many months and have obtained consistent results upon applying it to cultured neurons, with no evidence in changes in potency or effects. Regarding the possible causes of the effects *in vivo*, we cannot rule out the possibility that metabolites could contribute to some of the effects. Although there is fairly little known about BMAA metabolism, it appears that large amounts of BMAA can be recovered from tissues – especially in protein bound fractions – suggesting that much of it is *not* metabolized. However, there is evidence for some metabolism of BMAA that may differ between tissues, with certain metabolites appearing in liver and kidney, but not in brain tissue (see *Nunn and Ponnusamy, 2009 [Toxicon 54:85-94]*).

- over emphasis on the protein incorporation of L-BMAA in the Discussion -- the claim that L-BMAA is protein-incorporated has not been confirmed.

Although much is not known about mechanisms of BMAA toxicity *in vivo*, studies of BMAA isolation from bacterial, plant, animal and human tissues as discussed above seem to indicate that BMAA can be tightly and relatively stably associated with the protein fraction, such that levels recovered are dramatically increased after acid hydrolysis of the protein pellet (as first reported in *Murch et al. 2004a [PNAS 101: 12228-31]*). While there is only one paper as yet specifically reporting evidence of BMAA misincorporation into polypeptide chains (*Dunlop et al., 2012 [Plos One 8: e75376]*), we feel that this does represent a credible and potentially important mechanism to explain the long lasting protein association. Also consistent with such a mechanism, a recent study examined the time course of uptake of ^{14}C labeled BMAA into brain upon intravenous administration, and found the BMAA first to appear in the soluble fraction, but within hours begins to appear in the protein bound fractions, along with a progressive decrease in the soluble BMAA, suggesting that this was consistent with BMAA being trapped in proteins in a protein synthesis dependent fashion (see *Xie et al, 2014 [Neuroreport 24: 779-84]*).

We appreciate the reviewer's suggestion that this mechanism not be overstated or described as proven. In revising the current version, we have tried to give more background and description of the evidence in support of and significance of long lasting close protein association, but in our discussion of incorporation as a possible mechanism of the association, we are careful to make clear that this is only one possible mechanism that has not been proven to account for the association seen *in vivo*. To address more carefully these issues pertaining to long lasting

associations of BMAA with proteins, we have made modification and additions in the introduction (p. 3-4) and the discussion (p. 15-16) sections.

- *absence of cited references relating to work on L-BMAA excitotoxicity by authors other than those associated with this group.*

I agree! Although we have carried out a number of studies concerning excitotoxic mechanisms of BMAA toxicity over many years (having discovered its bicarbonate dependency for activating glutamate receptors, and having been the first to find it to cause selective injury to vulnerable subpopulations of neurons including MNs via actions at AMPA/kainate receptors at far lower concentrations than were needed to cause widespread NMDA receptor mediated injury) , there are a good number of others who have made important contributions, and I now try to provide a fuller review of earlier studies by multiple investigators as well as a new review highlighting evidence for and mechanisms of BMAA acting as an excitotoxin (see Discussion, p. 13-15).

Highlights:

- Prolonged intrathecal infusions of BMAA were carried out in WT and SOD1 mutant rats
- Pathological changes induced by BMAA were similar to those induced by mutant SOD1
- Changes were selectively observed in ventral horn with minimal dorsal horn changes
- Changes included MNs in different stages of degeneration with surrounding astrogliosis
- Findings may be compatible with contributions of BMAA in some human ALS

Intrathecal infusion of BMAA induces selective motor neuron damage and astrogliosis in the ventral horn of the spinal cord

Hong Z. Yin, MD [a], Stephen Yu [a], Cheng-I Hsu, PhD [a], Joe Liu [a], Allan Acab [a], Richard Wu [a], Anna Tao [a], Benjamin J. Chiang [a] and John H. Weiss, MD, PhD [a,b,*]

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Abbreviations: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA); amyotrophic lateral sclerosis (ALS); amyotrophic lateral sclerosis–Parkinsonism Dementia Complex of Guam (ALS/PDC); beta-N-methylamino-L-alanine (BMAA); Ca^{2+} permeable AMPA receptors (Ca-AMPA receptors); glutamate transporter 1 (GLT-1); motor neuron (MN); superoxide dismutase type 1 (SOD1); TAR DNA-binding protein 43 (TDP-43).

Abstract

The neurotoxin beta-N-methylamino-L-alanine (BMAA) was first identified as a "toxin of interest" in regard to the amyotrophic lateral sclerosis–Parkinsonism Dementia Complex of Guam (ALS/PDC); studies in recent years highlighting widespread environmental sources of BMAA exposure and providing new clues to toxic mechanisms have suggested possible relevance to sporadic ALS as well. However, despite clear evidence of uptake into tissues and a range of toxic effects in cells and animals, an animal model in which BMAA induces a neurodegenerative picture resembling ALS is lacking, possibly in part reflecting limited understanding of critical factors pertaining to its absorption, biodistribution and metabolism. To bypass some of these issues and ensure delivery to a key site of disease pathology, we examined effects of prolonged (30 day) intrathecal infusion in wild type (WT) rats, and rats harboring the familial ALS associated G93A SOD1 mutation, over an age range (80±2 to 110±2 days) during which the G93A rats are developing disease pathology yet remain asymptomatic. The BMAA exposures induced changes that in many ways resembles those seen in the G93A rats, with degenerative changes in ventral horn motor neurons (MNs) with relatively little dorsal horn pathology, marked ventral horn astrogliosis and increased 3-nitrotyrosine labeling in and surrounding MNs, a loss of labeling for the astrocytic glutamate transporter, GLT-1, surrounding MNs, and mild accumulation and aggregation of TDP-43 in the cytosol of some injured and degenerating MNs. Thus, prolonged intrathecal infusion of BMAA can reproduce a picture in spinal cord incorporating many of the pathological hallmarks of diverse forms of human ALS, including substantial restriction of overt pathological changes to the ventral horn, consistent with the possibility that environmental BMAA exposure could be a risk factor and/or contributor to some human disease.

Key words: BMAA; beta-N-methylamino-L-alanine; neurotoxin; ALS; amyotrophic lateral sclerosis; motor neuron; G93A rat; SOD1; animal model.

Introduction

Amyotrophic lateral sclerosis (ALS) is an adult onset neurodegenerative disease characterized by the selective loss of upper and lower motor neurons (MNs). A rare form of the disease called Guam ALS–Parkinsonism Dementia Complex (ALS/PDC) comprises ALS, often with associated symptoms of Parkinsonism and Alzheimer's like dementia, occurred in native Chamorro populations with an incidence >50 times that of ALS in most of the world (Arnold et al., 1953; Koerner, 1952; Mulder et al., 1954). Intensive epidemiological studies implicated environmental exposures (Garruto and Yase, 1986; Spencer, 1987), with early attention focusing on highly toxic cycad seeds, which were used for food after repetitive washing and cooking (Kurland, 1972; Whiting, 1963), and a neurotoxic non-protein amino acid, beta-N-methylamino-L-alanine (BMAA, also known as alpha-amino-beta-methylaminopropanoic acid) which they contained (Vega and Bell, 1967). Large oral doses of BMAA were reported to induce a syndrome in primates with features of ALS (Spencer et al., 1987), but the apparent low potency of BMAA and low levels found in washed cycad flour compared to amounts used in the study led to doubts to its relevance to human disease (Duncan et al., 1990).

Findings over the past decade have highlighted new sources for BMAA exposure and led to a resurgence of the BMAA hypothesis. It is now apparent that BMAA is produced by diverse taxa of cyanobacteria found worldwide (Cox et al., 2005; Metcalf et al., 2008), that BMAA appears to be either tightly associated with or incorporated into proteins of organisms that have been exposed to it, where it may reside for prolonged periods of time and be released by hydrolysis (Murch et al., 2004a), and that BMAA may be present in human tissues not only of Chamorros who died of Guam ALS/PDC but in tissues from sporadic ALS and Alzheimer's patients in North America and other regions (Murch et al., 2004b; Pablo et al., 2009). This, together with findings of high levels of protein associated BMAA in washed cycad flour as well as in food species present in aqueous environments prone to cyanobacterial blooms suggests that BMAA exposures outside of Guam may be far more widespread than previously believed (Bradley et al., 2013; Brand et al., 2010; Jonasson et al., 2010; Murch et al., 2004a). Indeed,

consistent with the apparent close protein association of BMAA, it appears that BMAA does bioaccumulate, with long-lasting residence in tissues and increasing levels often found at higher trophic levels of the food chain (Brand et al., 2010; Cox et al., 2003; Jonasson et al., 2010; Mondo et al., 2012). However, although BMAA is clearly neurotoxic with diverse effects in different model systems, an animal model reproducing key pathological features of ALS via systemic administration has been lacking (Karamyan and Speth, 2008).

In prior studies of BMAA toxicity to spinal cord neurons in culture, we found that BMAA was weakly toxic to most spinal neurons, but induced MN injury with far greater potency (Rao et al., 2006). Thus, the primary aim of the present study was to examine effects of prolonged BMAA exposure *in vivo*, to see if it could reproduce a more complete picture of ALS-like spinal cord pathology incorporating distinctive changes in other cell types, including astrocytes as well as in MNs themselves. In order to bypass variables and unknowns related to absorption, distribution and metabolism of BMAA *in vivo*, we opted to examine effects of direct delivery to the spinal cord, carrying out prolonged (30 day) intrathecal infusion of this toxin in both wild type rats, and in rats overexpressing the familial ALS associated G93A SOD1 mutation. We find that BMAA exposure induced changes in ventral horn MNs and in nearby astrocytes that were remarkably reminiscent of changes seen in untreated rats harboring the familial ALS associate G93A mutation in the Cu/Zn superoxide dismutase gene (SOD1), with manifestations in both models substantially localized to the ventral horn region of the spinal cord (where MNs reside), and markedly fewer changes noted in dorsal horn. Present studies thus indicate that with direct application, BMAA is capable of inducing spinal cord degenerative changes with similarities to those seen in both SOD1 linked animal models and human ALS, and are consistent with the hypothesis that BMAA is a contributory factor to some human ALS.

Materials and methods

Animals: Male hemizygous SOD1 G93A transgenic rats [Tac:N:(SD)-TgN(SOD1G93A)L26H, obtained from the Emerging Models Program sponsored by Amyotrophic Lateral Sclerosis Association, Taconic laboratories, Germantown, NY] were bred with wild-type females, and offspring genotyped by PCR analysis (Howland et al., 2002); wild-type siblings serve as controls for mutant animals. Animals are killed when they can no longer right themselves within 10 s of being pushed on their side. All animal procedures were approved by the Institutional Animal Care and Use Committee.

Surgical procedures: Intrathecal infusion studies used rats at 80±2 days of age (body weight 250g-380g). Anesthesia was induced using 5% isoflurane, and maintained at 2.5% at a flow rate of 1 l/min. Catheter and reservoir placement were carried out largely as previously described (Yin et al., 2007). Briefly, an incision was made in the dorsal head and the atlanto-occipital membrane, through which a PE5 catheter was inserted into the subarachnoid space and advanced 7–10 cm to the lumbar enlargement. The catheter was connected to an Alzet mini-osmotic pump model 2004; 200 µl volume, 0.25 µl/h × 30 days) which was pre-filled with 5mM BMAA or saline. After surgery, animals were housed individually and body weight recorded daily. In addition, evidence of pain or infection and motor dysfunction were closely monitored. Animals were sacrificed if they appeared distressed or if after 5 days, motor function remained impaired or body weight had not recovered to pre-surgery levels.

Tissue preparation and staining: Thirty days after the start of intrathecal infusions, the animals were anesthetized with isoflurane, and perfused transcardially with saline, followed by 4% paraformaldehyde (PFA) for 10 min. The lumbar enlargements of spinal cords were dissected and post-fixed in 4% PFA for 24 h and removed to 30% sucrose/PBS for another 2 days. Serial 20-µm frozen sections were cut from the middle of the lumbar enlargement for ~ 5 mm in the caudal direction.

After every 10 sections, 3–5 serial sections were set aside for staining. Immunohistochemical stains were carried out on floating sections, blocked (10% HS, 1 h), and exposed to primary antibody in 10% HS, 0.3% Triton-X 100 (SMI-32, 1:8000 IP, Sternberger Monoclonals, Berkeley, CA; GLT-1, 1:1000 IF, Chemicon, Temecula, CA; 3-nitrotyrosine, 10 μg/ml IF, Upstate Biotechnology, Waltham, MA; TDP-43, 1:10,000 IP, Proteintech Group, Chicago, IL). Labeling was visualized either by routine ABC immunoperoxidase techniques or under fluorescence using secondary antibodies linked to fluorophores (Alexafluor 488, Molecular Probes, Eugene, OR).

Quantification of histopathological changes: Surviving MNs were counted in ventral horn of lumbar spinal slices from each condition. MNs displaying pyknotic nuclei, marked atrophy or swelling, or fragmentation of the proximal dendrites were not counted as alive (Fig. 2).

For examination of 3-NT and GLT-1 labeling staining in the neuropil surrounding ventral horn MNs, care was taken to ensure that all slices from each experiment were labeled using identical simultaneous antibody exposures, and fluorescence photographs taken with identical camera settings so that labeling intensities could be compared. For quantification of GLT-1 labeling, photographs were imported into an image analysis package (Image J, public domain software from the NIH) as 8-bit gray scale images, and two regions were marked around each readily identifiable MN: one precisely outlining the circumference of the MN and another 10 um further out (with any other neurons or neuronal processes masked out). The space between these regions was defined as the ***surround zone***. After subtraction of background fluorescence, averaged intensity was calculated within each surround zone.

Chemicals and reagents: BMAA was obtained from Sigma (St. Louis, MO). Antibodies were from the following sources: SMI-32, Sternberger Monoclonals, Berkeley, CA; GLT-1, Chemicon, Temecula, CA; 3-nitrotyrosine, Upstate Biotechnology, Waltham, MA; TDP-43, Proteintech Group, Chicago, IL. For

fluorescence labeling, we used secondary antibodies linked to Alexafluor 488 (Molecular Probes, Eugene, OR). All other chemicals and reagents were obtained from common commercial sources.

Results

Intrathecal infusion of BMAA causes preferential MN injury

We set out to undertake a four-way comparison, examining pathological features of the lumbar spinal cord in wild type rats infused with saline only (WT+saline), or with BMAA (WT+BMAA), with features in age matched sibling G93A SOD1 transgenic rats infused with saline only (Tg+saline) or with BMAA (Tg+BMAA). In choosing the age for the study animals, we desired the SOD1 mutant animals to be pre-symptomatic, yet old enough that early pathological changes are evident. As these animals generally showed initial symptoms at ~ 140-160 days of age, we chose to start the intrathecal infusions at an age of 80±2 days, when early pathological changes are apparent (Howland et al., 2002; Yin et al., 2007), with sacrifice for histological analysis 30 days later (110±2 days). Catheters, connected to Alzet osmotic minipumps loaded with either saline alone or with 5 mM BMAA, were inserted in the cervical spine, and threaded intrathecally to terminate in the region of the lumbar enlargement as previously described (Yin et al., 2007) (see Materials and Methods).

MN degeneration was assessed in lumbar spinal cord sections with Nissl stain, and immunocytochemically using an antibody to non-phospholated neurofilament epitopes, SMI-32, which strongly labels MNs as well as their neuritic processes (Carriedo et al., 1996; Rao et al., 2006). In the WT+saline condition virtually all MNs appeared healthy. However, in the transgenics (Tg+saline), there were many MNs in differing stages of degeneration, manifested by a spectrum of changes including cellular and nuclear constriction, eccentric nuclei, swelling, vacuolar changes and fragmentation of processes. Many degenerating MNs were surrounded by microglia, with some appearing to be fully replaced by glial nodules and (Figs. 1, 2). In The WT+BMAA condition, a similar spectrum and frequency of MN degenerative changes was seen, with some MNs showing atrophic changes and some

showing more swelling with evident vacuolar changes. In addition, microglial infiltration and some microglial nodules were noted in this condition as well.

One question we were eager to address was whether BMAA infusion into the mutant SOD1 rat spinal cords (Tg+BMAA) would elicit synergistic injury. Leading us to consider it likely that we would see synergism, BMAA can induce neuronal injury via excitotoxic activation of glutamate receptors (Chiu et al., 2011; Vyas and Weiss, 2009), and mutant SOD1 has been reported to increase susceptibility to excitotoxic injury in both dissociated culture (Roy et al., 1998), and spinal cord slice culture (Yin and Weiss, 2012) models. However, in the present paradigm, we did not observe evidence of marked synergism, with evident MN degeneration in the Tg+BMAA condition not substantially different from that observed in WT+BMAA or Tg+saline conditions (Fig. 2).

A further pathological hallmark of MNs seen in most ALS cases is the presence of cytosolic protein inclusions. To assess possible cytosolic protein aggregation, we carried out immunostains for the peptide, TDP-43, which is normally present in the nucleus, where it has roles in RNA processing, and has been observed to leave the nucleus and to accumulate in cytosolic aggregates in diseased MNs in diverse forms of human ALS (Neumann et al., 2006). Interestingly, whereas such distinctive TDP-43 pathology is not a prominent early feature of rodent SOD1 mutant ALS models (Robertson et al., 2007; Turner et al., 2008), it has been observed in damaged MNs in these animals at advanced stages of the disease (Shan et al., 2009). For these studies, we used an antibody against the N-terminal region of TDP-43, expression of which is normally quite strongly restricted to the nucleus, as we found to be the case in virtually all MNs in the WT+saline condition. However, loss of distinct nuclear TDP-43 labeling with appearance of some cytosolic staining was noted in a fraction of the damaged and degenerating MNs in the other three conditions, with little difference noted in either the frequency or appearance of cytosolic TDP-43 labeling between them (Fig. 3).

Pathological changes in astrocytes

Immunostains employing three different antibodies were undertaken to examine distinct aspects of astrocyte pathology. First, reactive astrogliosis was assessed using the astrocyte marker, glial fibrillary acidic protein (GFAP). Ventral horn astrogliosis is a prominent feature of human ALS, and progressive astrogliosis occurs prominently in SOD1 mutant rodent models of ALS, including the G93A rat model examined in these studies (Howland et al., 2002; Yin et al., 2007). Notably, astrocyte pathology in both human disease and the SOD1 mutant rodent models is highly restricted, with prominent changes in the ventral horn astrocytes which are adjacent to disease affected MNs, with a paucity of changes in dorsal horn astrocytes until late stages of the disease.

In the control (WT+saline) condition, most GFAP labeled astrocytes in the ventral horn were small and faintly labeled. In contrast, WT+BMAA, Tg+saline, and Tg+BMAA conditions each showed substantial increases in both numbers of labeled astrocytes as well as in their size and intensity of labeling in ventral horn. Interestingly, dorsal horn astrocytes appeared little different from control in the WT+BMAA and Tg+saline conditions, but, although not quite statistically significant over the set of 5-8 animals studied, appeared in most animals to be modestly increased in both number and labeling intensity in the Tg+BMAA condition, possibly compatible with some degree of synergism between effects of the SOD1 mutation and the BMAA that is not readily evident under the present exposure paradigm (Fig. 4).

Oxidative tissue damage, and particularly increases in labeling for the oxidative damage marker, 3-nitrotyrosine (3-NT), occurs in both human and SOD1 mutant rodent models of ALS (Beal et al., 1997; Ferrante et al., 1997; Yin et al., 2007). Immunolabeling for 3-NT reveals increased labeling in the ventral horn compared to surrounding white matter pathways, which is evident even in the WT+saline condition. Although we and other have previously found strong increases in ventral horn 3-NT labeling in late presymptomatic G93A SOD1 mutant rats compared to WT rats, we observed relatively little difference between the WT+saline and Tg+saline conditions here, likely reflecting the age of the animals compared to the time of disease onset (~110 days, with symptoms appearing at ~140-165 days). However, the

presence of BMAA (in both WT+BMAA and Tg+BMAA conditions) resulted in a marked increase in ventral horn 3-NT labeling, which, much as we have previously described in the presymptomatic G93A mutant animals (Rao and Weiss, 2004; Rao et al., 2003; Yin et al., 2007), was particularly evident in the neuropil surrounding and between MNs (Fig. 5), consistent with the idea that MN dysfunction contributes directly to the 3-NT labeling in adjacent astrocytes.

The third marker of astrocyte pathology we examined was the astrocytic glutamate transporter, GLT-1. Loss of astrocytic glutamate transport capacity, resulting specifically from loss or dysfunction of the GLT-1 transporter, is seen in human ALS (Rothstein et al., 1992; Rothstein et al., 1995), a finding that lent initial strong support to the idea that excitotoxic activation of glutamate receptors might contribute to MN damage in the disease. Furthermore, loss of GLT-1 occurs prominently in the G93A rats employed in this study (Howland et al., 2002; Yin et al., 2007). As has been previously noted, labeling of control slices for the astrocytic glutamate transporter, GLT-1, shows diffuse staining throughout the spinal cord gray matter. However, a rim of particularly strong labeling is often seen immediately surrounding MN somata, which we have previously found be markedly decreased even in presymptomatic SOD1 mutant rats (Yin et al., 2007). To quantify these changes, we measured the intensity of GLT-1 labeling in 10 μm zones surrounding identified MNs in the spinal cord slices as described (see Materials and Methods). We found that BMAA induced significant decreases in the intensity of GLT-1 labeling in the surround zones that was nearly as great as those induced in the Tg+saline and Tg+BMAA conditions (Fig. 6).

Discussion

Summary of principal findings

We find that 30 day intrathecal infusions of BMAA induce a distinctive pathological picture in the spinal cord, which shows striking similarities to that seen in rats harboring the G93A SOD1 mutation. Notably, despite being an exogenously applied toxin, which should have equal access to ventral horn and dorsal horn tissues within the spinal cord, the pathological changes induced by BMAA infusions were

reminiscent of those seen in the mutant SOD1 rats, with a strong preponderance of changes seen in the ventral horn region, where the MNs reside. Interestingly, BMAA and mutant SOD1 each induced a similar spectrum of pathological changes among the MNs, including some MNs demonstrating swelling and vacuolar changes, while others showed prominent atrophic changes, and some were surrounded by microglia or replaced by microglial nodules. In addition, as noted in the SOD1 mutant rats, the BMAA infusion resulted in loss of nuclear TDP-43 labeling with speckled accumulation in cytosol of some of the damaged and degenerating MNs.

Distinctive changes resembling those in the SOD1 mutant rats were also noted in the astrocytes and neuropil surrounding damaged ventral horn MNs, with prominent astrogliosis, as well as increases in 3-NT labeling, and a loss of GLT-1 from the region immediately surrounding MNs. Surprisingly, in the present paradigm, we noted relatively little synergism between the effects of BMAA and of the G93A mutation, with ventral horn changes induced by BMAA infusion in G93A mutant rats little different from those caused by either alone. The only suggestion of synergism was in the case of GFAP staining in the dorsal horn, where, although not reaching statistical significance, in most animals numbers of GFAP labeled astrocytes was modestly greater in the Tg+BMAA condition. The reasons for the paucity of apparent synergism is uncertain, but perhaps synergistic effects would become more evident either pathologically or clinically with greater durations of co-exposure.

Clues to mechanisms of MN vulnerability in ALS

Despite a large number of studies and numerous clues, the basis of the selective vulnerability of MNs in ALS is poorly understood. As discussed above, findings of loss of astrocytic glutamate transport in ALS supported an "excitotoxic" contribution (Rothstein et al., 1992). Glutamate activates distinct families of ionotropic receptors, including the highly Ca^{2+} permeable NMDA receptors and the generally Ca^{2+} impermeable AMPA and kainate receptors. Whereas most studies of excitotoxic neurodegeneration have thus focused on contributions of NMDA receptors, some AMPA receptors gate channels that are directly

permeable to Ca^{2+} ("Ca-AMPA receptors"), reflecting absence of the GluA2 (formerly called GluR2) subunit in heteromeric channels. MNs possess large numbers of these unusual Ca-AMPA receptors, making them highly sensitive to AMPA receptor mediated injury (Carriedo et al., 1995; Carriedo et al., 1996; Van Den Bosch et al., 2000; Vandenberghe et al., 2000). Another factor that may contribute to MN vulnerability is that they have low levels of cytosolic Ca^{2+} binding proteins (Alexianu et al., 1994; Elliott and Snider, 1995), causing them to buffer cytosolic Ca^{2+} poorly (Lips and Keller, 1998; Vanselow and Keller, 2000), such that cytosolic Ca^{2+} loads are rapidly taken up into mitochondria, resulting in mitochondrial dysfunction and ROS generation (Carriedo et al., 2000; Rao et al., 2003). Based upon these observations, we have suggested that the high susceptibility of MNs to degeneration in disease may in part reflect a high propensity to oxidative stress. Whereas astrocyte dysfunction is a prominent feature in ALS and clearly contributes to the MN damage, its causes are poorly understood. In light of our findings that excitotoxic MN activation causes strong mitochondrial ROS generation, and the documented susceptibility of glutamate transport to oxidative disruption, we considered, and found evidence in support of, an hypothesis that ROS generated in MNs in response to Ca-AMPA receptor activation can contribute to oxidative dysfunction in surrounding astrocytes (Rao and Weiss, 2004; Rao et al., 2003), possibly contributing to the increased 3-NT labeling and the and loss of GLT-1 labeling surrounding MNs we observed in G93A rats (Yin et al., 2007).

Another prominent feature of ALS is neuroinflammation, manifesting both cellular and humoral responses (Philips and Robberecht, 2011; Zhao et al., 2013). Activation of both astrocytes and microglia occur prominently, and activated microglia invade and contribute to phagocytosis of damaged MNs. The neuroinflammation also results in production of cytokines, which also can contribute to the MN damage. Of note the potent cytokine TNF-α can act on MNs to increase numbers of cell surface Ca-AMPA receptors, and thus their susceptibility to excitotoxic injury (Ferguson et al., 2008; Yin and Weiss, 2012), possibly accelerating the injury cascade. It is intriguing that BMAA infusions appear to reproduce astrocytic and microglial involvement resembling that seen in the SOD1 mutant rodent models. The

mechanisms of this are uncertain, but in addition to the possibility discussed above that MN ROS generation contributes to astrocyte activation, MN damage may release antigens that contribute to the triggering of inflammatory responses. It is also possible that BMAA can induce effects directly on astrocytes, via a number of possible receptor dependent or independent mechanisms as discussed further below.

A distinct mechanism that is strongly suspected of contributing to MN degeneration in ALS concerns the accumulation of cytosolic protein aggregates (Blokhuis et al., 2013), reflecting an increased propensity for aggregate production in MNs, or defects in the ability to clear aggregates. In line with this idea, cytosolic protein aggregates are seen in all forms of ALS (with cytosolic TDP-43 aggregates specifically seen in most forms whether or not associated with mutations in the TDP-43 gene). Oxidative stress, as we suggest may occur prominently in MNs in response to excitotoxic Ca^{2+} loads, could promote protein misfolding and aggregate formation. Conversely, a family of ALS associated genes has been recently uncovered all of which normally function in protein degradative and autophagic pathways (Fecto and Siddique, 2011), suggesting that deficiencies in ability to get rid of aggregated proteins and/or dysfunctional organelles (including mitochondria) could also be important contributors to disease. An important intracellular target of protein aggregates may be mitochondria, dysfunction in which is seen in all forms of ALS, and in particular mutant SOD1 aggregates are clearly documented to associate with and contribute to dysfunction of mitochondria (Manfredi and Xu, 2005).

Clues to mechanisms of BMAA neurotoxicity

A number of studies of BMAA, mainly carried out using *in vitro* systems, have highlighted mechanisms through which BMAA may mediate neurotoxicity (Chiu et al., 2011; Vyas and Weiss, 2009). BMAA is an atypical non-protein amino acid. The first indication that it might act through excitotoxic mechanisms were provided by the observations that it could cause convulsions in rats (Polsky et al., 1972), and that it caused postsynaptic vacuolar changes in neurons similar to other excitotoxins (Nunn et al., 1987).

Although early studies suggested that it caused excitotoxic tissue injury via weak activation of NMDA receptors (Kd ~ 1 mM in 1 day exposure) (Ross et al., 1987), it lacks the side-chain acidic or electronegative moiety characteristic of other excitatory amino acid compounds, having instead a positively charged amine group, leading to the suggestion the mechanism through which it activated glutamate receptors might be indirect (Nunn et al., 1987; Ross et al., 1987).

Providing a possible explanation for neuroexcitatory effects of BMAA, we found that BMAA could only activate glutamate receptors if bicarbonate was present in the extracellular buffer (Weiss and Choi, 1988). The presence of bicarbonate / CO_2 in the buffer results in the formation of carbamate adducts on the side chain amino groups (Myers and Nelson, 1990; Nunn and O'Brien, 1989), likely resulting in a structure resembling glutamate, in which the positively charged amine is replaced by an acidic group (Vyas and Weiss, 2009; Weiss et al., 1989a); and many subsequent studies have found evidence for excitotoxic effects of BMAA that are presumed to reflect the presence of the carbamate adduct; for a review see (Chiu et al., 2011).

A second question concerned the receptors through which BMAA mediates excitotoxic injury. Although BMAA is a weak agonist at NMDA receptors, we found that it caused selective degeneration of a subpopulation of cortical neurons ("NADPH-diaphorase" neurons) at far lower concentrations (30-100 μM) than needed for it to induce widespread damage via NMDA receptor activation, and that it mediated this selective injury via an AMPA rather than an NMDA receptor mechanism (Weiss et al., 1989b). Indeed, this finding, taken together with identification of 2 other environmental motor system toxins that acted through AMPA/kainate receptor mechanisms led us to undertake studies (discussed above) demonstrating the presence of Ca-AMPA receptors on MNs as a factor underlying an unusual susceptibility to AMPA receptor mediated injury (Carriedo et al., 1995; Carriedo et al., 1996; Van Den Bosch et al., 2000; Vandenberghe et al., 2000). We subsequently examined the vulnerability of MNs in dissociated spinal cord cultures to BMAA mediated neurotoxicity, and found that MNs were indeed selectively injured by BMAA, with 30-100 μM levels causing substantial MN degeneration over 24

hours, while mM levels were needed to cause widespread neuronal damage. Furthermore, the selective MN degeneration was entirely blocked by the AMPA antagonist, NBQX, indicating that the MN degeneration was mediated through these receptors (Rao et al., 2006).

Other recent studies have highlighted additional ways in which BMAA might contribute to neurodegeneration. First, in addition to actions at NMDA and AMPA receptors described above, BMAA can activate metabotropic glutamate receptors (Copani et al., 1991). Intriguing recent studies suggest that micromolar levels of BMAA may be able to enhance diverse forms of neuronal injury (Lobner et al., 2007) through mechanisms including interference with function of the cysteine /glutamate antiporter (system Xc(-)) leading to glutathione depletion and increased oxidative stress, while promoting excess glutamate release (Liu et al., 2009). Metabolites of BMAA could also contribute to some of its effects (Nunn and Ponnusamy, 2009).

Another important issue concerns the ability of BMAA to get across the blood brain barrier and into the central nervous system (CNS), and its possible interactions with or incorporation into proteins, accounting for it apparent persistence in the CNS. As discussed above, BMAA has been isolated from tissues many years after the times of likely exposure, and levels recovered are greatly increased after acid hydrolysis, suggesting either incorporation or close association with proteins. Although early studies revealed that with systemic administration, some BMAA passes the blood brain barrier and enters the brain (Kisby et al., 1988) and that with repeated large oral dosage, free BMAA levels in rat brain can reach levels in the 100's of micromolar (Duncan et al., 1991), a recent study in rats examining the time course of brain BMAA uptake shows free soluble BMAA to appear first in the brain, with protein bound BMAA starting to accumulate within hours of administration, possibly consistent with its incorporation (Xie et al., 2013). An *in vitro* study recently found evidence for BMAA incorporation into proteins in place of L-serine (Dunlop et al., 2013), providing support for the hypothesis that incorporation could underlie the apparent long residence of BMAA in tissue protein fractions. Indeed, whatever the precise mechanism, long lasting association of BMAA with proteins may be critical to its apparent

bioaccumulation in the food web, and provides a basis for BMAA to act as a slow neurotoxin, mediating toxicity for prolonged periods after the time of exposure, and accounting for the likely long latency between exposures and any occurrence of neurodegenerative disease. This slow toxicity could potentially reflect effects on proteins themselves as well as effects of free BMAA, which may be slowly released from the protein reservoir upon protein turnover.

Conclusions

It is interesting and somewhat surprising that intrathecal infusions of BMAA can induce a pathological picture in spinal cord that in a number of ways – both involving degenerative changes in the MNs themselves, and changes in the neuropil and astrocytes in the ventral horn region surrounding the MNs – resembles that seen in the G93A mutant SOD1 overexpressing rat model as well as in diverse forms of human ALS. Indeed, it is notable that most human ALS shows a distinctive pathological phenotype despite the growing number of unrelated genes from distinct gene families that have been associated with genetic forms of the disease – suggesting that the characteristic pathological "picture" of ALS results in large part from intrinsic features of MNs and their interdependencies with associated cells and environmental factors. Thus, in the case of BMAA induced pathological changes, the "picture" most likely reflects a combination of toxic mechanisms of BMAA superimposed upon the predisposition of the affected tissues.

As discussed above, prior studies have highlighted a number of ways in which BMAA is likely to promote pathophysiologically relevant mechanisms of MN degeneration. First, as we have found in culture models, exposures in the 10's of μM range can trigger relatively selective MN injury via activation of Ca-AMPA receptors which are strongly expressed on MNs. In addition to directly damaging MNs, such excitotoxic activation of MNs by BMAA could, via mechanisms including induction of oxidative stress, contribute to the dysfunction of surrounding astrocytes. Although steady state levels of BMAA in the lumbar spinal fluid during present intrathecal infusions are unknown, the osmotic pump

used releases ~ 0.25 µl/hr of 5 mM BMAA, which, if distributed in a local spinal CSF volume of ~ 250 µl (dilution of 1:1000/hr), and with a several hour half-life for CSF turnover, might yield steady state BMAA levels in the ~10-30 µM, which *in vitro* studies have suggested may well cause slow preferential MN damage. In addition, suggestions that BMAA may be misincorporated into or otherwise stably associated with proteins provides a potentially attractive mechanism to explain the long residence of BMAA in tissues and latency after exposure before disease onset, while possibly promoting protein misfolding and aggregation, as occurs in and has been considered likely to play pathogenic roles in neurodegenerative diseases including ALS (Blokhuis et al., 2013).

In sum, it is apparent that there is a wide spectrum of causes of ALS, with a number of clearly genetic causes triggered by mutations in distinct and unrelated genes from different gene families, as well as strong evidence for environmental contributions to the disease. In light of recent findings that BMAA is widespread in the environment, appears to be present in tissues of some human disease patients, and can contribute to the induction of a characteristic pathological phenotype (as seen here), likely acting through mechanisms (excitotoxicity, oxidative stress, protein misfolding) that are known to occur in ALS, might BMAA be an important toxin of interest to the disease meriting further investigation (Bradley et al., 2013)?

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Figure Legends

Figure 1. *Thirty day BMAA infusions induce a similar spectrum of morphological changes in rat ventral horn MNs as is seen in presymptomatic rats harboring the G93A SOD1 mutation.* G93A SOD1 mutant rats and sibling WT rats were subjected to 30 day intrathecal infusions of BMAA or saline (from 80±2 to 110±2 days of age) as described, followed by perfusion, sectioning of the lumbar spinal cords and Nissl staining to examine cellular morphology. For each treatment (as indicated), a low power image of a representative hemi-section of the lumbar spinal cord (40x) is shown, and details (VHa and DHa) show high power (400x) views of the marked regions of the ventral horn and the dorsal horn respectively. VHb and VHc show other representative high power views of ventral horn sections from the same treatment. In contrast to the healthy appearing MNs in the WT+saline condition, MNs in the Tg+saline condition displayed a spectrum of degenerative morphologies including cell swelling and vacuolar chages (thin arrows), MN shrinkage (thick arrows), and microglial infiltration or glial nodule formation (arrowheads). A similar spectrum of changes was noted in the WT+BMAA and Tg+BMAA conditions. In contrast to the marked changes noted in ventral horn MNs, little evidence of dorsal horn neuronal damage was noted in any of the conditions. Scale bar = 400 μm (low power panels) or 50 μm (all other panels).

Figure 2. *Morphological changes and quantification of lumbar ventral horn MN degeneration induced by the G93A SOD1 mutation and by 30 day BMAA infusions.* G93A mutant SOD1 and sibling WT rats were subjected to 30 day intrathecal infusions with BMAA or saline as described, followed by perfusion, sectioning of the lumbar spinal cords and SMI-32 immunostaining for counts of surviving MNs. SMI-32 labeling provides excellent visualization of somatic and dendritic morphology and is thus useful for assessing structural damage.

TOP: *Morphological changes*. For each treatment (as indicated), a low power image of a representative SMI-32 stained hemi-section of the lumbar spinal cord (40x) is shown, along with a high power (400x)

view of the marked region of ventral horn. As in Figure 1, above, note the intact somatic and dendritic morphology in the control condition (WT+saline), and the mix of somatic changes with some MNs showing atrophy (thick arrows) and some showing swelling (thin arrows), as well as widespread fragmentation of dendritic processes in the other conditions. Scale bar = 500 μm (low power panels) or 50 μm (all other panels).

BOTTOM: Quantification of MNs cell loss. Healthy appearing intact MNs were counted in SMI-32 immunostained slices from each condition. Values represent mean number of intact ventral horn MNs per section (comprising two ventral horns), as assessed by direct microscopic examination. Each data point reflects the mean value from 5-9 animals (WT+BMAA, 9; WT+saline, 6; Tg+saline, 5; Tg+BMAA, 5 animals); all ventral horn MNs were counted in each of 18-23 slices from each animal, with a total of 1700-3500 MNs counted in each condition. *indicates difference from WT animals by two tailed t-test ($p<0.001$).

Figure 3. *The G93A SOD1 mutation and 30 day BMAA infusions both induce the appearance of cytosolic TDP-43 immunoreactive aggregates in some MNs.* G93A mutant SOD1 and sibling WT rats were subjected to 30 day intrathecal infusions with BMAA or saline as described, followed by perfusion, sectioning of the lumbar spinal cords and immunostaining for TDP-43. Note that the TDP-43 labeling in large MNs is distinctly nuclear in the WT+saline condition, whereas in the other conditions, some of the large MNs (indicated by arrows) show disruption of homogeneous nuclear labeling and accumulation of cytosolic aggregates. Scale bar = 100 μm.

Figure 4. *The G93A SOD1 mutation and 30 day BMAA infusions both induce prominent reactive astrogliosis in the ventral horn.* G93A mutant SOD1 and sibling WT rats were subjected to 30 day intrathecal infusions with BMAA or saline as described, followed by perfusion, sectioning of the lumbar spinal cords and immunostaining for GFAP.

TOP: For each treatment (as indicated), images show representative high power (200x) immunofluorescence views from ventral horn (VH) and dosal horn (DH) regions of the spinal cord gray matter. Note that compared to the control (WT+saline) condition, all of the other conditions show a marked increase in number and intensity of GFAP labeled reactive astrocytes in the ventral horn. Further note that GFAP labeling is not increased in the dorsal horn, with the possible exception of the Tg+BMAA condition, in which there appeared to be a slight increase in GFAP labeling. Scale bar=100 μm.
BOTTOM: Quantification of GFAP labeling. Reactive astrocytes were counted in GFAP immunolabeled slices from each condition. Values represent mean number of distinct GFAP-positive astrocytes per unit area in 200x microscope fields. Each data point represents the mean value from 5 to 8 animals; for each animal all GFAP immunoreactive astrocytes were counted in each of 6 ventral horn and dosal horn sections (with ~ 500-1500 cells counted for each condition). * indicates difference from VH in WT+saline condition ($p<0.02$); # indicated difference from VH in the same condition ($p<0.05$) by two-tailed t test.

Figure 5. *Effects of the G93A SOD1 mutation and of 30 day BMAA infusions on 3-NT labeling in ventral horn.* G93A mutant SOD1 and sibling WT rats were subjected to 30 day intrathecal infusions with BMAA or saline as described, followed by perfusion, sectioning of the lumbar spinal cords and immunostaining for 3-NT. For each treatment (as indicated), images show representative high power (200x) immunofluorescence views from 3-NT immunolabeled slices. Inserts show the same regions displayed using pseudocolor (8 bit) representations of fluorescence, in order to highlight the gradient in labeling intensity. Note the moderate labeling of WT MNs, and the clear increased labeling in the WT+BMAA and Tg+BMAA conditions not only in the MNs, but prominently in the neuropil surrounding and between MNs. Scale bar = 100 μm.

Figure 6. *The G93A SOD1 mutation and 30 day BMAA infusions each induce loss of the astrocytic glutamate transporter, GLT-1, in regions surrounding ventral horn astrocytes.* G93A mutant SOD1 and sibling WT rats were subjected to 30 day intrathecal infusions with BMAA or saline as described, followed by perfusion, sectioning of the lumbar spinal cords and immunostaining for GLT-1.

TOP: For each treatment (as indicated), images show representative 200x immunofluorescence views from GLT-1 immunolabeled slices; details show blow-ups of the regions marked by arrowheads. Note the strong GLT-1 labeling often seen immediately surrounding ventral horn MNs in the WT+saline condition, in contrast to the marked loss of GLT-1 labeling surrounding ventral horn MNs in the other conditions. Scale bar = 100 µm (low power), 50 µm (high power images),

BOTTOM: Quantification of GLT-1 labeling intensity in 10 µm wide zones sourrounding MN somata. Values represent the mean fluorescence compared to control (WT+saline =100%) in the surround regions from 5-9 animals for each condition, based on measurements from 10-12 ventral horns sections per animal and a total of 500-1000 MN surrounds measurements in each conditions. * indicates difference from WT+saline by two-tailed t test ($p<0.002$).

Figure 1

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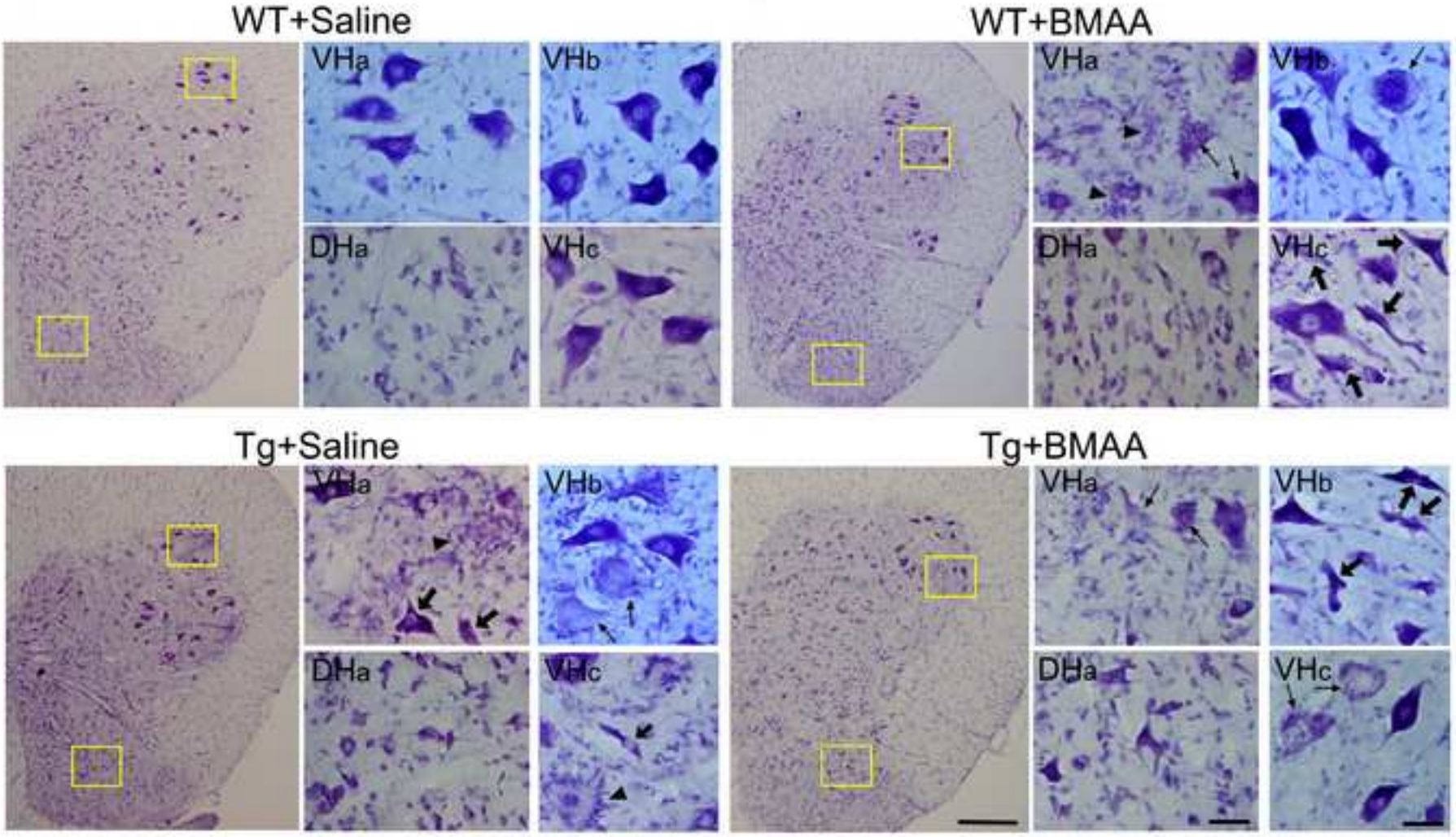

Figure 2

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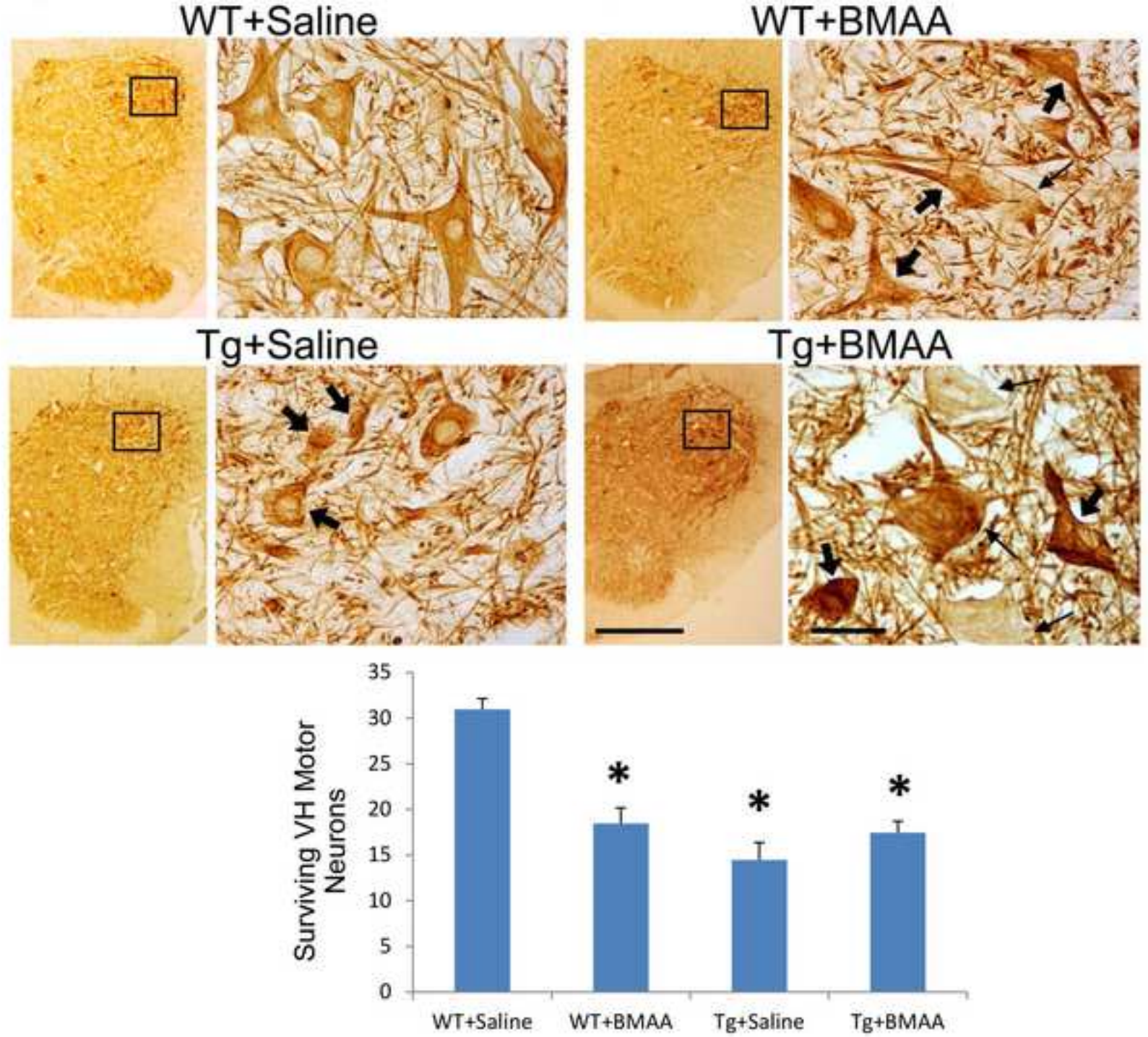

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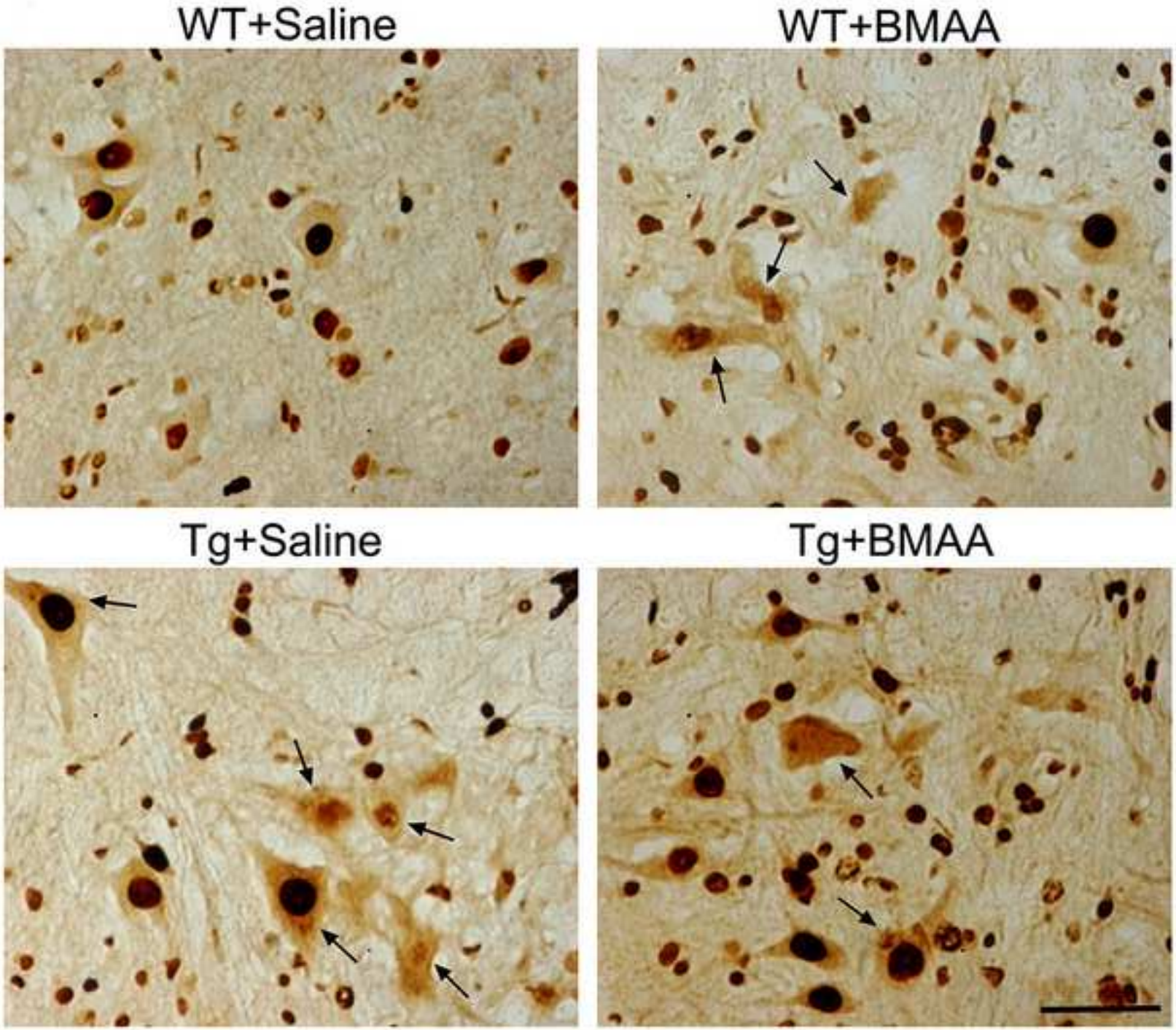

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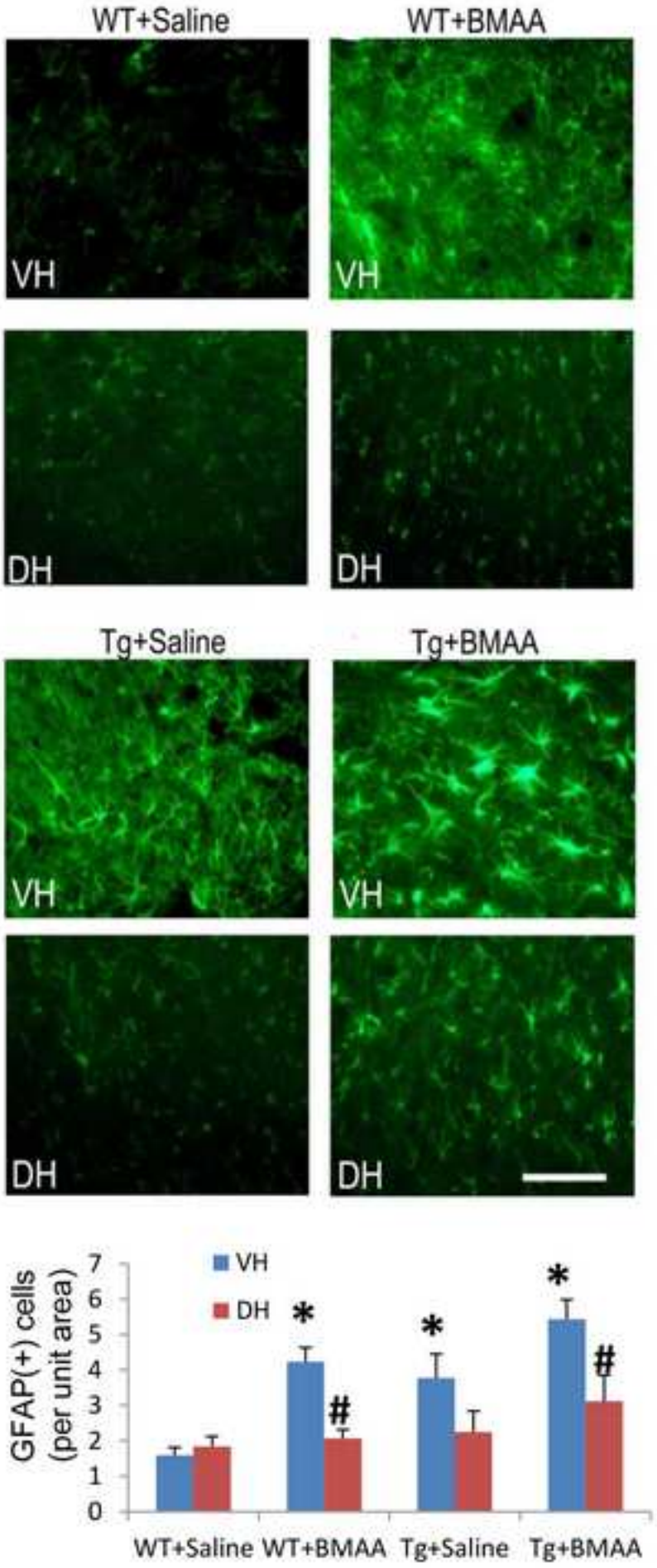

Figure 5

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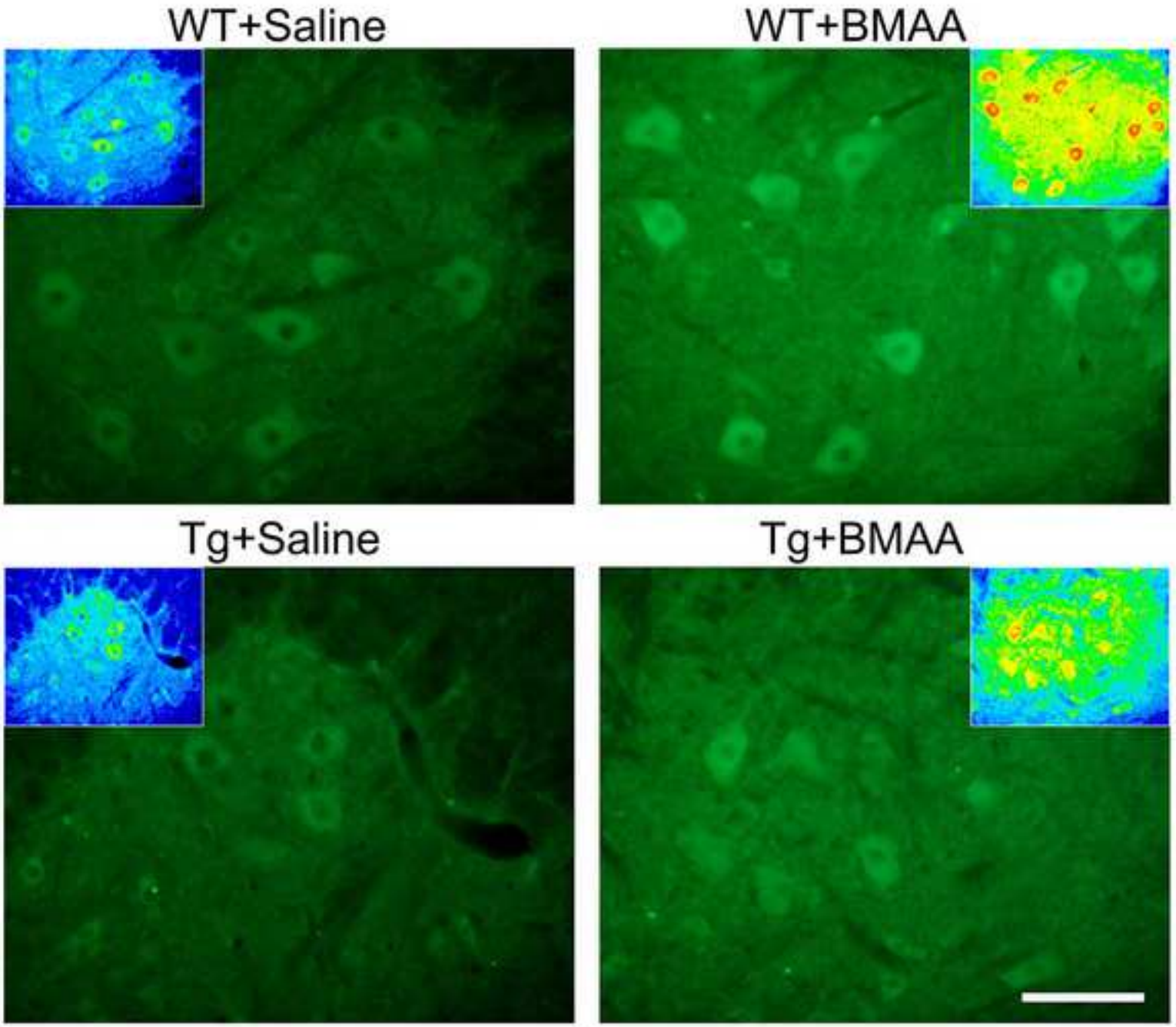

Figure 6

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