

Halogenated aromatic amino acid 3,5-dibromo-D-tyrosine produces beneficial effects in experimental stroke and seizures

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Abstract The effects of the halogenated aromatic amino acid 3,5-dibromo-D-tyrosine (3,5-DBr-D-Tyr) were studied in rat models of stroke and epileptic seizures caused by middle cerebral artery occlusion (MCAo) through respective intracerebral injection of endothelin-1 (ET-1) and intraperitoneal (i.p.) injection of pentylenetetrazole (PTZ). 3,5-DBr-D-Tyr was administered as three bolus injections (30 or 90 mg/kg, i.p.) starting at 30, 90, and 180 min after ET-1 administration or as a single bolus (30 mg/kg, i.p.) 15 min prior to PTZ administration. Neurological deficits and infarct volume were estimated 3 days after ET-1 administration and seizure score was assessed during the first 20 min after PTZ administration. The safety of 3,5-DBr-D-Tyr was evaluated in control animals using telemetry to measure cardiovascular parameters and immunostaining to assess the level of activated caspase-3. 3,5-DBr-D-Tyr significantly improved neurological function and reduced infarct volume in the brain even when the treatment was initiated 3 h after the onset of MCAo. 3,5-DBr-D-Tyr significantly depressed PTZ-induced seizures. 3,5-DBr-D-Tyr did not cause significant changes in arterial blood pressure, heart rate and spontaneous

locomotor activity, nor did it increase the number of activated caspase-3 positive cells in the brain. We conclude that 3,5-DBr-D-Tyr, by alleviating the deleterious effects of MCAo and PTZ in rats with no obvious intrinsic effects on cardiovascular parameters and neurodegeneration, exhibits promising potential as a novel therapeutic direction for stroke and seizures.

Keywords Ischemic stroke · 3,5-DBr-D-Tyr · PTZ-caused seizures · Arterial blood pressure · Caspase-3

Introduction

Stroke continues to be a major cause of death, disability, and economic expense worldwide. This problem is made worse by an aging population. The only effective therapy to date is reperfusion of ischemic brain regions within hours of symptom onset. Both the narrow time window for reperfusion and risk of intracranial hemorrhage severely limits the number of patients eligible for treatment. Practically all of the pharmacological options for stroke treatment proposed to date failed when tested in clinical settings (Dirnagl and Macleod 2009). Therefore, the search for novel therapeutic modalities is an important agenda for both basic and clinical science.

The pathophysiology of stroke is a complex process with excessive activation of glutamate signaling (excitotoxicity), mediated by the *N*-methyl-D-aspartate (NMDA), (RS)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors, as one of the primary and early events in the mechanisms underlying stroke-induced brain damage (Mehta et al. 2007). In severe cases of stroke this excitotoxicity sometimes manifests as a generalized seizure. On the other hand, glutamate is a major excitatory

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neurotransmitter that plays a crucial role in normal brain physiology (Hardingham 2006; Hetman and Kharebava 2006). Because all elements of the glutamatergic system are functionally linked and, therefore, are all involved in hyperexcitability and neurotoxicity, antiglutamatergic agents with polyvalent actions are more likely to produce clinically desirable effects. This is because such agents may depress glutamatergic activity to levels that retain the balanced glutamate receptor activity required for physiological brain functions, thus avoiding significant side effects.

During investigation of the cellular mechanisms whereby high concentrations of aromatic amino acid phenylalanine (Phe) affect the brain in phenylketonuria (PKU) patients, we found that L-Phe depresses excitatory glutamatergic transmission in a way that may form a basis for the development of drugs for the treatment of pathophysiological conditions for which hyperactivated excitatory signaling plays an important etiological role. These diseases include stroke, seizures, and many chronic neurodegenerative disorders. Specifically, L-Phe, at concentrations observed in PKU brain, significantly depresses excitatory glutamatergic synaptic transmission by a combination of pre- and postsynaptic actions: (1) inhibition of NMDA receptor activity; (2) depression of AMPA/kainate receptors; and (3) attenuation of neurotransmitter release (Glushakov et al. 2002, 2003, 2005). The halogenated derivative of 4-hydroxyphenylalanine (tyrosine), 3,5-dibromo-D-tyrosine (3,5-DBr-D-Tyr), produces more potent polyvalent antiglutamatergic effects (Martynyuk et al. 2006). In this study 3,5-DBr-D-Tyr, administered after the onset of stroke, produced efficacious neuroprotection with no obvious side effects. In addition, 3,5-DBr-D-Tyr was effective at diminishing seizures in a rat model of epileptic seizures caused by pentylentetrazole.

Materials and methods

Animals

All experimental procedures were approved by the University of Florida Institutional Animal Care and Use Committee. In addition, the principles governing the care and treatment of animals, as stated in the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (publication No. 85-323, revised 1996) were followed at all times during this study. Male Sprague-Dawley rats (6–8 week old, 250–300 g) were housed in groups of two at room temperature on a 12 h light/dark cycle.

Middle cerebral artery occlusion procedure

Ischemic strokes were induced by intracerebral injection of endothelin-1 (ET-1) adjacent to the middle cerebral artery as described in detail previously (Sharkey et al. 1993; Mecca et al. 2009; Cao et al. 2009). In brief, the skull of the anesthetized animals was exposed and a small hole was drilled in the cranium dorsal to the right hemisphere using the following stereotaxic coordinates (1.6 mm anterior and 5.2 mm lateral to the bregma). A 26-gauge needle attached to a Hamilton microsyringe was lowered to a depth of 8.7 mm and 3 μ l of saline solution containing 240 pmol of ET-1 was injected at a rate of 1 μ l/min. The syringe was left in place for 4 min, before removal and wound closure. Rats were treated in a randomized fashion with three i.p. injections of 3,5-DBr-D-Tyr of 30 or 90 mg/kg each or equal volumes of saline with the first injection performed at 30, 90, or 180 min after intracerebral administration of ET-1 (see “Results” for details). The sunflower seed eating task (Gonzalez and Kolb 2003) and the neurological exams originally described by Garcia et al. (1995) were performed 72 h following the MCAo to evaluate neurological function of the rats as described in detail previously (Cao et al. 2009). Infarct volume was determined by integrating infarct areas of sequential brain slices and expressed as percentage of homologous tissue volume of the contralateral hemisphere as described previously (Cao et al. 2009). Tissue in brain sections that was not stained with 2,3,5-triphenyltetrazolium chloride was assumed to be infarcted.

Pentylentetrazole-induced seizures

Pentylentetrazole (PTZ, 60 mg/kg, i.p.) was used to induce seizures in rats that were pretreated 15 min earlier either with 3,5-DBr-D-Tyr (30 mg/kg, i.p.) or an equal volume of saline. The rats were observed and videotaped for 20 min after the administration of PTZ. The severity of seizures was scored using a six-point behavioral seizure score: 0, no response; 1, ear and facial twitching; 2, convulsive waves through the body; 3, myoclonic jerks and/or rearing; 4, clonic-tonic seizures; 5, generalized clonic-tonic seizures, loss of postural control.

Measurements of cardiovascular parameters and locomotor activity in conscious rats

Heart rate, mean arterial blood pressure, pulse pressure, and locomotor activity were recorded using rat telemetry transducers (DSI, St. Paul, MN, USA) as described previously (Cao et al. 2009).

Activated caspase-3 determination

The whole brains were isolated and immediately frozen in liquid nitrogen and stored at -80°C . Two 20- μm -thick coronal sections of the whole brain from the same anatomical location for all groups of rats were cut on a cryostat (Leica Microsystems LM3050S) and mounted on poly-L-lysine-coated slides (Richard Allen, Kalamazoo, MI). The following sets of primary antibodies were used for immunostaining: rabbit anti-human activated caspase-3 IgG (polyclonal, 1:100; Cell Signaling Technology, Danvers, MA), mouse anti-neuronal nuclei (NeuN) IgG (monoclonal, 1:100; Chemicon International, Temecula, CA) to identify neurons, and anti-glia fibrillary acidic protein (GFAP; monoclonal, 1:600; Sigma, St. Louis, MO) to localize astrocytes. The following fluorescent secondary antibodies were used: AlexaFluor 594-conjugated goat anti-rabbit (Molecular Probes, Eugene, OR) or FITC-conjugated goat anti-mouse (Zymed Laboratories, San Francisco, CA). To test for nonspecific staining by the secondary antibodies, additional slides were processed in a similar fashion with the primary antibodies excluded. An Olympus microscope (Olympus Corp., Tokyo, Japan) was used to review the tissue sections after processing. Co-localization of immunoreactivity was performed with Photoshop (Adobe Systems; San Jose, CA) to superimpose paired images showing the fluorescent secondary antibodies identifying anti-active caspase-3 and the cell type of interest.

Statistical analysis

For parametric data, single comparisons were tested using the *t* test, whereas multiple comparisons among groups were analyzed using one-way or two-way repeated measures ANOVA followed by Bonferroni *t* test. For non-parametric data, the Mann–Whitney rank sum test was used. $P < 0.05$ was considered significant. SigmaStat 3.11 software (Systat Software, Inc, Point Richmond, CA) was used for statistical analysis.

Results

Anti-seizure effects of 3,5-DBr-D-Tyr in a rat model of PTZ-induced seizures

First, we tested whether 3,5-DBr-D-Tyr can be efficient at depression of epileptic seizures caused by PTZ in vivo. Adult male Sprague-Dawley rats were randomly distributed into two groups with similar numbers of animals of the same age in both groups. The animals in group one received 3,5-DBr-D-Tyr (30 mg/kg, i.p.) 15 min prior to

administration of PTZ. Rats in group two received equal volumes of saline. The animals' seizure responses were analyzed for 20 min following administration of PTZ. The change in rat's behavior caused by 60 mg/kg PTZ (i.p.) ranged from no response to generalized clonic-tonic seizures. Responses of rats pre-treated with 3,5-DBr-D-Tyr were characterized by a significantly increased latent period before the start of seizures (Fig. 1a). In addition, both the duration (Fig. 1b) and severity (Fig. 1c) of seizures in the 3,5-DBr-D-Tyr-pretreated group were significantly decreased.

Neuroprotective effects of 3,5-DBr-D-Tyr in a rat model of stroke elicited by intracerebral injection of ET-1

3,5-DBr-D-Tyr was administered as three equal bolus injections (i.p.) following intracerebral injection of ET-1

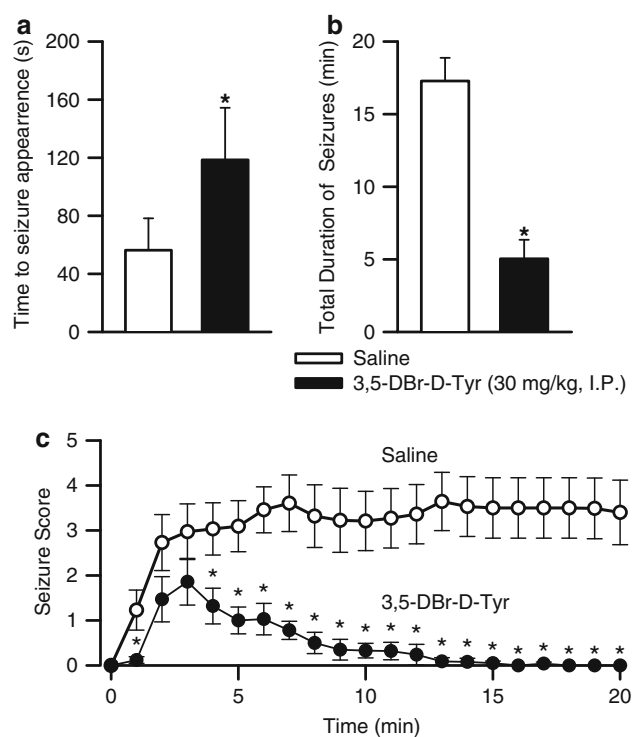
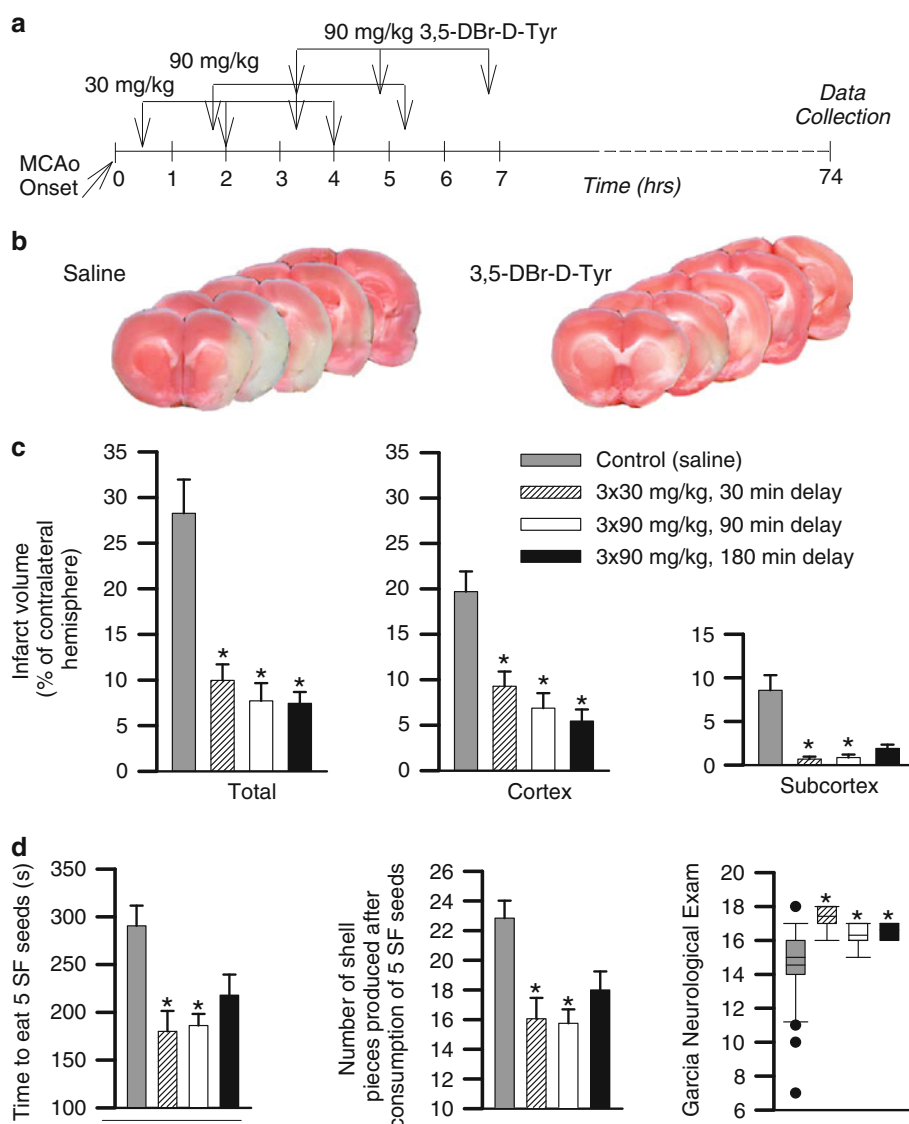


Fig. 1 3,5-DBr-D-Tyr depresses PTZ-induced seizures in rats. Each group of animals received an injection of either 3,5-DBr-D-Tyr (30 mg/kg, i.p., $n = 8$) or equal volumes of saline ($n = 10$) 15 min prior to PTZ administration (60 mg/kg, i.p.). The bar graphs show the effects of 3,5-DBr-D-Tyr on time delay for seizures appearance after PTZ administration (a) and on total seizure duration during the 20-min observation period (b), $*P < 0.05$ versus control. c Seizure intensity at a given time. The seizure intensity during bin intervals of 1 min was characterized using a six-point behavioral score: 0, no response; 1, ear and facial twitching; 2, convulsive waves through the body; 3, myoclonic jerks and/or rearing; 4, clonic-tonic seizures; 5, generalized clonic-tonic seizures, loss of postural control. $*P < 0.05$ seizure intensity at each time point in the 3,5-DBr-D-Tyr-treated rats versus corresponding time point in the saline-treated animals

into the area of the middle cerebral artery to cause transient vessel occlusion (stroke). Treatment with the lower dose of 3,5-DBr-D-Tyr (30 mg/kg) was initiated 30 min after the administration of ET-1, while treatments with higher doses of 3,5-DBr-D-Tyr (90 mg/kg) were started either 1.5 or 3 h after the ET-1 injection. In each treatment schedule the second and third boluses of 3,5-DBr-D-Tyr were administered 1.5 and 3.5 h after initial drug injections (Fig. 2a). Rats in the control group received equal volumes of saline following the same schedule. Neurological and anatomical evaluations were performed 3 days following the induction of stroke. Neurological symptoms and infarct measurements showed significant deleterious effects of stroke in the control group of rats (Fig. 2b–d). Both, neurological deficits and volumes of the injured brain tissue were

significantly diminished in animals treated with 3,5-DBr-D-Tyr (Fig. 2) when compared with the same parameters in the saline-treated animals. The therapeutic effects of 3,5-DBr-D-Tyr depended on the latency period between ET-1 administration and initiation of treatment. 3,5-DBr-D-Tyr significantly improved the scores of all neurological tests and decreased infarct volume both in cortex and subcortex when the treatment was initiated at 30 and 90 min after administration of ET-1. When treatment was delayed for 3 h, on the other hand, the score for the Garcia neurological exam and the stroke volume in the cortex were still significantly improved, while improvements in the scores of the sunflower seed test and stroke volumes in the subcortex were not sufficient to achieve statistical significance.

Fig. 2 3,5-DBr-D-Tyr decreases the volume of infarcted brain tissue and the extent of the neurological deficit caused by intracerebral injection of ET-1. 3,5-DBr-D-Tyr was administered intraperitoneally as three boluses, 30 mg/kg each or 90 mg/kg each, starting 30 min ($n = 15$), 90 min ($n = 12$), and 180 min ($n = 8$) after the onset of MCAo at times shown in **a**. **b** 2,3,5-triphenyltetrazolium chloride-stained sections of brain at five coronal levels from representative rats that received either saline or 3,5-DBr-D-Tyr (30 mg/kg). Control animals received equal volumes of saline. **c** Volumes of infarcted brain tissue presented as percent of contralateral hemisphere. **d** The results of the sun flower (SF) seed test and Garcia neurological exams in the same groups of animals. Histopathological and neurological evaluations were performed 3 days after administration of ET-1. All control data from three animal groups were combined and presented as one group (gray bar). * $P < 0.05$ versus 0.9% Saline. Box and whisker plots the boundaries of the box indicate the 25th and 75th percentile; the line within the box marks the mean. Whiskers (error bars) indicate the 90th and 10th percentiles



3,5-DBr-D-Tyr neither causes cell apoptosis nor affects blood pressure, heart rate, and general activity in rats

Here we assessed whether 3,5-DBr-D-Tyr at doses that were used to induce neuroprotection from injuries caused by intracerebral administration of ET-1 may cause neurotoxicity on its own. One half of the rats received 3,5-DBr-D-Tyr (30 mg/kg, i.p.) as three consecutive boluses at 0, 90, and 210 min. Rats in a control group received equal volumes of saline following the same time schedule. No MCAo procedure was performed in these animals. Eighteen hours after the end of 3,5-DBr-D-Tyr or saline administrations the animals were killed and apoptotic changes in the brain were determined by evaluating activated caspase-3 using immunostaining. As shown in Fig. 3, the levels of activated caspase-3 immunoreactivity in the cortex of the 3,5-DBr-D-Tyr- and saline-treated animals were similar.

In order to investigate the effects of 3,5-DBr-D-Tyr on cardiovascular parameters and general activity, rats that did not undergo MCAo received 3,5-DBr-D-Tyr using the same dose and timing protocol for drug administration as in the caspase-3 experiments (see above). The recordings started

2 h prior to 3,5-DBr-D-Tyr administrations. Therefore, each animal served as its own control. As shown in Fig. 4, injections of 3,5-DBr-D-Tyr transiently increased blood pressure, heart rate, and activity in the rats. However, all parameters quickly returned to pre-treatment levels soon after injection procedures. These increases were probably due to the stress caused by animal handling during the injection procedures rather than the drug by itself as their magnitude decreased with each successive administration; similar changes were seen during 0.9% saline injections in our previous study (Cao et al. 2009).

Discussion

This study demonstrates that stroke-impaired neurological functions were improved by 3,5-DBr-D-Tyr, administered after the onset of stroke. The improvement in neurological functions was also associated with decreased histological brain damage in these animals. In addition, 3,5-DBr-D-Tyr diminished PTZ-induced seizures. The absence of significant changes in cardiovascular parameters and activated caspase-3 levels in the 3,5-DBr-D-Tyr-treated rats indicates that this agent is not likely to produce the typical side effects caused by selective glutamate antagonists. Taken together, these findings support the premise that 3,5-DBr-D-Tyr is a promising candidate for the acute treatment of ischemic stroke and epileptic seizures.

3,5-DBr-D-Tyr was devoid of clinically significant systemic cardiac and hemodynamic effects. This makes a direct effect on regional cerebral blood flow as part of its neuroprotective actions unlikely. Previously, we have shown that another halogenated derivative of aromatic amino acids, 3,5-dibromo-L-phenylalanine (3,5-DBr-L-Phe) depressed PTZ-induced seizures in rats and alleviated deleterious effects of intracerebral administration of ET-1 (Cao et al. 2009). Although the neuroprotective effects of equal doses of 3,5-DBr-D-Tyr and 3,5-DBr-L-Phe were not significantly different, treatment with 3,5-DBr-D-Tyr yielded better results for each studied parameter when compared with the vehicle-treated animals. Thus, improvement of all neurological functions and decrease of infarct volume were significant in animals treated with 3,5-DBr-D-Tyr, while the effects of 3,5-DBr-L-Phe were not significant for the time spent eating sunflower seeds and also for the volume of the infarcted tissue in the subcortex region (Cao et al. 2009). We have previously demonstrated that 3,5-DBr-D-Tyr and 3,5-DBr-L-Phe both depress glutamatergic activity but differ in mechanism of action (Yarotsky et al. 2005; Martynyuk et al. 2006). Both agents decrease glutamate release and depress AMPA/kainate receptor activity. The major difference is that 3,5-DBr-L-Phe acts as a partial agonist at the NMDA receptor producing 30% of the

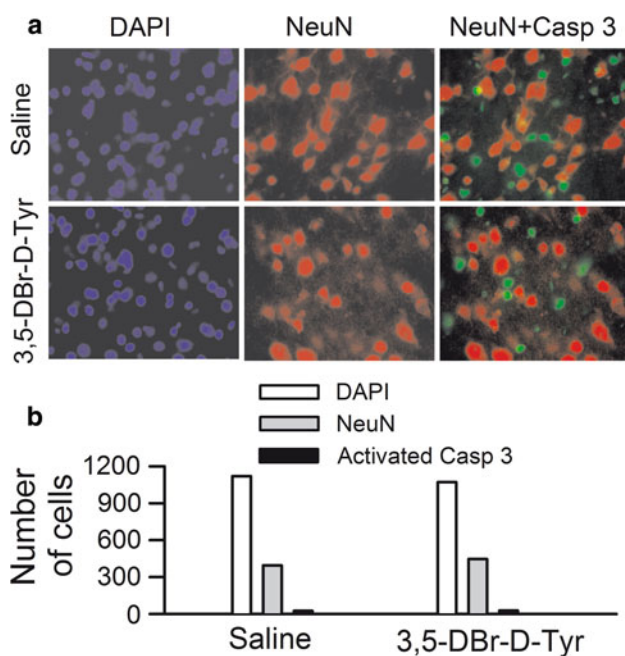
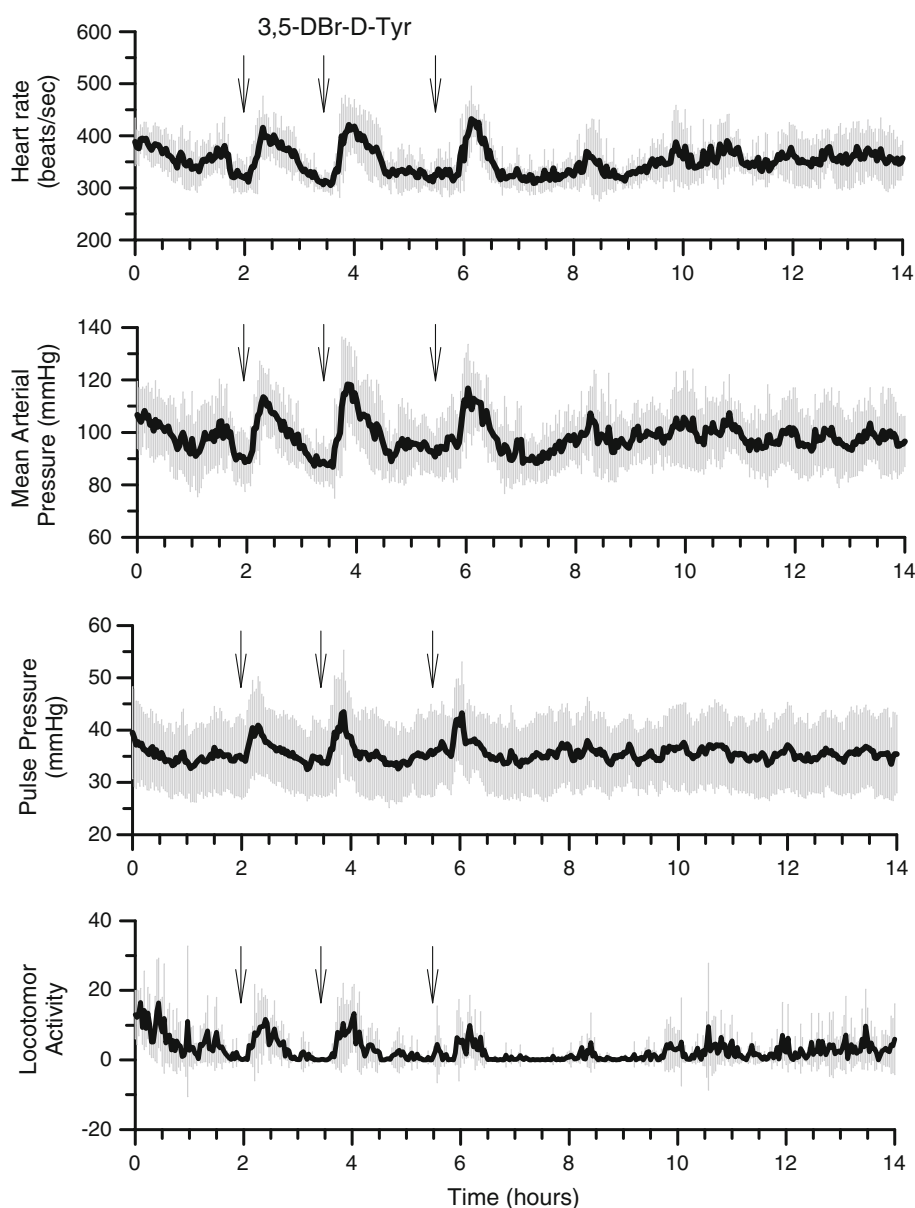


Fig. 3 3,5-DBr-D-Tyr does not change level of activated caspase-3 in the rat cortex. **a** Representative 40 $\times$ images of the cortices of the rats not exposed to middle cerebral artery occlusion: DAPI, NeuN and an overlay of NeuN and caspase-3 images. **b** The results of cell counting. Double immunolabeling of NeuN and activated caspase-3 do not show any detectable activated caspase-3 labeling in neurons of the cortices of the 3,5-DBr-D-Tyr-treated rats. Rats received three boluses i.p. of 3,5-DBr-D-Tyr 30 mg/kg each or equal volume of saline at 0, 90, and 210 min. Immunohistochemical analysis has been performed 18 h later

Fig. 4 3,5-DBr-D-Tyr does not significantly alter cardiovascular parameters and locomotor activity in the rats. Adult male SD rats, fitted with telemetry pressure transducers, received three bolus i.p. injections of 3,5-DBr-D-Tyr 30 mg/kg each at 120, 270, and 330 min after start of recordings and recordings continued another 12 h. *Arrows* indicate time points at which drugs were administered. Data are means (black circles) $\pm$ SE (gray bars) from six rats for each treatment group



maximal response of a full agonist, while 3,5-DBr-D-Tyr depresses NMDA receptor activity. The decrease in the depressant effect of 3,5-DBr-D-Tyr on the NMDA activated current with the increase of the extracellular concentration of NMDA indicates that 3,5-DBr-D-Tyr may act as a competitive inhibitor at the glutamate-binding site of the NMDA receptors (Martynyuk et al., 2006). If the anti-glutamatergic action of 3,5-DBr-D-Tyr and 3,5-DBr-L-Phe is an important component of their neuroprotective effects following ischemic stroke, then 3,5-DBr-D-Tyr should be more suitable for neuroprotection due to its pure antagonistic action at NMDA receptors. The action of 3,5-DBr-D-Tyr as an antagonist of NMDA receptors may also explain its more consistent effect among animals within the same treatment group. Activation of NMDA receptors during

brain ischemia may be different in different brain regions; for example, more potent in cortex and weaker in the subcortex. This could be one of the reasons why 3,5-DBr-L-Phe produced a significant decrease in volume of infarcted brain tissue in the cortex, but not in the subcortex (Cao et al. 2009). Involvement of the anti-glutamatergic actions of 3,5-DBr-D-Tyr and 3,5-DBr-L-Phe in mediating their neuroprotective effects is also supported by their overall similar but subtly different depressant action on epileptic seizures caused by PTZ. 3,5-DBr-L-Phe, which depresses presynaptic glutamate release with a sixfold greater potency than 3,5-DBr-D-Tyr, also depressed seizures more effectively (Cao et al. 2009).

Important properties of aromatic amino acids, that may contribute to the observed neuroprotective action of

3,5-DBr-D-Tyr, are their ability to trap $\cdot\text{OH}$ and ONOO^- . $\cdot\text{OH}$ is the most reactive oxygen metabolite in the cerebrum. In conditions characterized by elevated levels of reactive oxygen species, $\cdot\text{OH}$ reacts with Phe, primarily yielding 2-hydroxy(*o*-tyrosine), 3-hydroxy(*m*-tyrosine), and 4-hydroxyphenylalanine (*p*-tyrosine) (Biondi et al. 2006), while hydroxylation of Tyr results in 2,3- and 3,4-dihydroxyphenylalanine (dopa) (Exner et al. 2003). These amino acids could also be hydroxylated by $\cdot\text{OH}$ derived from the decomposition of peroxynitrite (van der Vliet et al. 1994). In addition, peroxynitrite may nitrate Phe and Tyr to nitro-Phe and nitro-Tyr (Murrant and Reid 2001; Park et al. 2003). Because of its close structural similarity, 3,5-DBr-D-Tyr is likely to share these properties of its parent molecules.

In addition to its actions at glutamate synapses and antioxidant properties, 3,5-DBr-D-Tyr like Phe may have differential effects on NR2A- and NR2B-containing NMDA receptors that could contribute to its neuroprotective action. Indeed, we have found that in the hyperphenylalaninemic PKU mouse brain the surface expression of NMDA receptor subunits NR2A and NR2B were significantly increased and decreased, respectively (Glushakov et al. 2005). Recently, several groups reported that inhibition of NR2A-containing NMDA receptors increased NMDA-induced apoptosis, whereas inhibition of NR2B-containing NMDA receptors was neuroprotective (Chen et al. 2008).

An important question that may have a decisive role in whether 3,5-DBr-D-Tyr or compounds with similar patterns of actions would have a therapeutic application is whether these compounds cause adverse effects that may preclude their clinical applicability. In support of their safety, 3,5-DBr-D-Tyr, in contrast to selective NMDA receptor antagonists, did not increase neuronal caspase-3 activation. Also, administration of 3,5-DBr-D-Tyr caused only transient, clinically nonsignificant changes in heart rate and arterial blood pressure. Those increases were probably due to a stress response from the injections as they were also previously seen in response to saline injections (Cao et al. 2009).

In summary, the halogenated aromatic amino acid 3,5-DBr-D-Tyr produced efficacious neuroprotection in rat models of stroke caused by MCAo and epileptic seizures caused by PTZ. Importantly, significant neuroprotection with 3,5-DBr-D-Tyr could be achieved by administering these agents hours after the onset of stroke. Taken together with our previous findings with 3,5-DBr-L-Phe, we speculate that the polyvalent balanced antigitamatergic action of 3,5-DBr-D-Tyr in particular, and certain halogenated derivatives of the aromatic amino acids, in general, provide significant neuroprotection via attenuation of glutamatergic activity that still allows close to normal physiological

function of this system, and, therefore, avoids significant side effects. 3,5-DBr-D-Tyr or agents with a similar mode of action may become novel, much needed drugs, or prototypes of drugs, not only for stroke, but also for other pathologies in which overactivated glutamatergic transmission plays an etiological role, such as epileptic seizures, traumatic brain injuries, and a number of neurological disorders.

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