The psychoactive drug 25B-NBOMe recapitulates rhabdomyolysis in zebrafish larvae

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Abstract N-Benzyl-substituted 2C class phenethylamines (NBOMes) are psychoactive designer drugs, with strong hallucinogenic and stimulant effects, even at low doses. The designer drug, 2-(4-bromo-2, 5-dimethoxyphenyl)-N-(2-methoxybenzyl) ethanamine (25B-NBOMe) is considered to be one of the most potent agonists of the serotonin-2A (5-HT2A) receptor. Recently, we reported the first lethal case of 25B-NBOMe intoxication with severe rhabdomyolysis, concluded by clinical, pathological and toxicological analyses. There are currently no good animal models that closely recapitulate serotonin receptor-dependent rhabdomyolysis. In the present study, we created animal models of rhabdomyolysis using zebrafish larvae to study the pathomechanism of rhabdomyolysis, and demonstrated that 25B-NBOMe can simulate lethal rhabdomyolysis in this animal. Treatment of the larvae with 25B-NBOMe decreased their survival rate, locomotion, altered birefringence of the skeletal muscle and immunostainings for dystroglycan (a myoseptal protein) and myosin heavy chain (a myofibril protein), which were consistent with rhabdomyolysis. This 25B-NBOMe-induced rhabdomyolysis was inhibited by the 5-HT2A receptor antagonists ritanserin and aripiprazole, but not by the 5-HT1A + 5-HT1B receptor antagonist propranolol and the 5-HT3 receptor antagonist granisetron, indicating 5-HT2A-dependent rhabdomyolysis. The 25B-NBOMe-treated zebrafish is, therefore, a highly useful model of rhabdomyolysis for studying the pathomechanism of rhabdomyolysis as well as for therapeutic drug screening.

Keywords 25B-NBOMe intoxication · Rhabdomyolysis mechanism · 5-HT2A receptor · Zebrafish larvae · Animal model · New psychoactive substance

Introduction

Serotonin syndrome is caused by the excessive activation of serotonin (5-hydroxytryptamine: 5-HT) receptors in the nervous system, and is characterized by autonomic hyperactivity, mental-status changes, and neuromuscular abnormalities including rhabdomyolysis [1, 2]. Serotonin syndrome is known to be induced not only by diverse serotonergic drugs [i.e., monoamine oxidase (MAO) inhibitors, selective serotonin reuptake inhibitors (SSRIs) and the 5-HT precursor (L-tryptophan)] and their combinations, but also by illegal drugs such as methylenedioxymethamphetamine (MDMA) [3]. In rodent models, a combination of serotonergic drugs (precursors) can induce serotonin receptor 2A (5-HT2A)-dependent hyperthermia [4]. However, to our knowledge, there has been no reproducible animal model of serotonin syndrome with overt rhabdomyolysis established to date.

N-2-Methoxy-benzyl substituted 2C class hallucinogens (NBOMes) had emerged as psychoactive designer drugs that potently activated 5-HT2A receptors [5]. NBOMe poisoning can cause various symptoms that are similar to serotonin syndrome, including tachycardia, hypertension, agitation, hallucinations, seizures, hyperpyrexia, and myoclonus [6]. Because 45% of the lethal cases of serotonin syndrome presented high serum levels of creatine kinase, rhabdomyolysis may be a predominant cause of...
death in NBOMe intoxication [7]. Recently, we reported a
case of serotonin syndrome with lethal rhabdomyolysis after
the ingestion of 2-(4-bromo-2,5-dimethoxyphenyl)-N-(2-methoxybenzyl) ethanamine (25B-NBOMe), one of the
most potent 5-HT2A agonists with a1-adrenoceptor agonist activity [8]. Prominent rhabdomyolysis was also reported
in two other cases with severe 25B-NBOMe intoxication
[9]. Because the patho-physiological mechanism underlying
rhabdomyolysis associated with serotonin syndrome is
largely unknown, we hypothesized that 25B-NBOMe will
contribute to the study of the mechanism of 5-HT2A-de-
pendent rhabdomyolysis.

Zebrafish have been widely used in neuroscience
researches due to their high genetic homology to humans,
genetic tractability, low cost, and, hence, usefulness in
high-throughput analyses [10]. Serotonergic drugs and
their combinations can induce serotonin syndrome-like
behavior such as surface dwelling and hypo-locomotion in
zebrafish, in association with high serotonin levels in the
brain [10]. In zebrafish larvae, the structures of organs,
such as the heart and skeletal muscle, can be observed
in situ due to their transparency and small size. Furthermore,
they can be analysed in a short period and in large
scale owing to their easy maintenance and housing, fast
growth, and high fecundity. Because of the in vivo visi-
bility of their skeletal muscle and easy genetic manipula-
tion, zebrafish has been used to study muscular dystrophy
[11–13] as well as for therapeutic drug screening [14–16].

Here, we present a novel, simple and reproducible model of 5-HT2A-dependent rhabdomyolysis induced by
25B-NBOMe in zebrafish.

Materials and methods

Chemicals

The 25B-NBOMe hydrochloride was purchased from Cayman
chemical (Ann Arbor, MI, USA); ritanserin, aripiprazole,
granisetron hydrochloride, and propranolol hydrochloride from
Sigma-Aldrich (St. Louis, MO, USA). Other common chemi-
cals used were of the highest purity commercially available.

Fish and fish culture

The Experimental Animal Committee of Tokyo Medical
University approved all experiments performed in this
study (approval number: S28029). Adult zebrafish (the AB
line) strains of both sexes were obtained from the Aquatic
Resources Program (Boston Children’s Hospital, Bos-
ton, MA, USA) and acclimatized to the laboratory envi-
ronment for at least 14 days in a 100-L aquarium filled
with continuously unchlorinated water at 28.5 °C, with
constant filtration and density of up to five animals per liter
according to the standard procedures [17] and standard
criteria [18]. The animals were kept on a day/night cycle of
12/12 h and fed twice a day with flaked fish food. Fertilized
eggs were collected and cultured for drug treatments.

Drug treatment of zebrafish

Pairs of adult AB zebrafish were mated, and their embryos
were cultured to larvae 4 days post fertilization (dpf).
Five-4-dpf larvae were put into one well (a total of three wells for
each condition) and were treated with either 0, 0.005, 0.5, or
5 μg/mL of 25B-NBOMe [stock solution: 10 mg/mL in
dimethyl sulfoxide (DMSO)] for 2 days to determine the
effectiveness of concentration of 25B-NBOMe. The survival rates
of 25B-NBOMe-treated and vehicle (DMSO)-treated fish
were analyzed. All experiments were repeated three times.

To analyze the 5-HT receptor subtype responsible for
effects, zebrafish larvae were treated with 25B-NBOMe
(0.5 μg/mL) and 5-HT receptor subtype inhibitors including
ritanserin (1.0 μM), aripiprazole (0.5 μM), granisetron
hydrochloride (100 μM), or propranolol hydrochloride
(10 μM) for 2 days. The concentration of each inhibitor was
set at the maximum concentration required to enable the sur-
vival of larvae for 2 days. Ritanserin is a selective
5-HT2A + 5-HT2C receptor antagonist; aripiprazole is a 5-HT2A
receptor antagonist, and partial 5-HT1A + dopamine D2
receptor agonist; granisetron hydrochloride is a 5-HT3 receptor
antagonist; propranolol hydrochloride is a 5-HT1A + 5-HT1B
antagonist and non-selective β-adrenoceptor (β-AR)
blocker. For the specificity of 5-HT subtype receptors, see
review [19]. All experiments were repeated four times.

Detection of muscle structural changes
by the birefringence assay

To monitor structural changes in skeletal muscle, birefrin-
gence was examined using a dissection microscope
(MZ10F; Leica Microsystems GmbH, Wetzlar, Germany).
Zebrafish larvae were anesthetized with 0.32% tricaine
solution, placed on a polarizing filter and covered with a
second polarizing filter. The filters were placed on a stere-
omicroscope and the top-polarizing filter was twisted until
only the light refracting through the striated muscle became
visible. As the degree of birefringence is affected by the
horizontal orientation of the fish, the fish were oscillated
back and forth to account for differences in positioning.
Muscle damage was detected as reduced birefringence.

Immunohistochemistry

For immunohistochemical staining, whole larvae were fixed
in 4% paraformaldehyde overnight at 4 °C and stored in
100% methanol at −20 °C. Following rehydration with a 50% methanol solution in phosphate buffered saline (PBS) containing 0.05% Tween 20 (PBS-T) and blocking with 2% casein in PBS-T to reduce non-specific immunoreactions, larvae were incubated with either anti-beta dystroglycan (1:100, Novoceastra; Leica Biosystems, Wetzlar, Germany) or anti-myosin heavy chain (MHC) (F59, 1:25; Santa Cruz Biotechnology, Dallas, TX, USA) antibodies at 4 °C overnight. After washing several times, the larvae were incubated with a secondary antibody (1:500, anti-mouse AlexaFluor 488; Thermo Fisher Scientific, Waltham, MA, USA) for 30 min at room temperature. The stained larvae were then observed using a confocal microscope (LCM710; Carl Zeiss Microscopy GmbH, Jena, Germany).

Behavioral analysis of zebrafish larvae

Each zebrafish larva of 4 dpf was put into each well of a 96-well plate containing the drugs and their swimming behavior was recorded for 5 min using Danio Vision (Noldus, Wageningen, The Netherlands). The swimming distance of each fish was measured using Danio Vision following the manufacturer’s instructions. Eight larvae were examined for each condition.

Reverse transcription polymerase chain reaction

To confirm the expression of the 5-HT2A receptor, zebrafish total RNA was extracted from the brain and skeletal muscle of adult zebrafish (3-month old) using the RNeasy micro kit (Qiagen, Venlo, The Netherlands) and was converted to cDNA (Superscript III; Thermo Fisher Scientific). To detect PCR products of 5-HT2A receptor cDNA, PCR was performed using ExTaq DNA Polymerase (Takara Bio, Kusastu, Japan) at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s for 35 cycles) with the following primer sets [20]: zebrafish 5-HT2A receptor, forward 5'-GCCACCAATTACTTCTCATGTCAC-3', reverse 5'-GGTTCA-CAAAACCCTCGCCAAAC-3'; and β-actin, forward: 5'-ATCAGCATGGCTCTGCTCT-3', reverse: 5'-CACCTGGTGTCTTCTTCTTCA-3'. The resulting DNAs were subjected to gel electrophoresis for visualization of DNA bands corresponding to 5-HT2A receptor and β-actin [20].

Results

Reduction of zebrafish larva survival by 25B-NBOMe

From 4 dpf, zebrafish larvae were treated with 25B-NBOMe at the concentrations of 0, 0.005, 0.5, or 5 μg/mL for 2 days. Treatment with 25B-NBOMe was found to decrease the survival rate of zebrafish in a dose-dependent manner (Fig. 1a). Treatment with 0.5 μg/mL of 25B-NBOMe resulted in a reduction in survival rate to about 60% of the control fish (untreated: 93.3 ± 9.4, 0.005% DMSO: 95.6 ± 8.3%, 25B-NBOMe at 0.5 μg/mL: 58.3 ± 15.2%). Therefore, in subsequent experiments, we used 25B-NBOMe at 0.5 μg/mL (Fig. 1a, p = 0.00000458).

Muscle degeneration in 25B-NBOMe-treated larvae

Two days after the treatment of zebrafish larvae with 0.5 μg/mL of 25B-NBOMe, the ratio of live larvae with...
reduced birefringence was 65–80% (25B-NBOMe at 0.5 μg/mL: 66.0 ± 20%, Fig. 1c). Immunofluorescence with an anti-beta dystroglycan antibody demonstrated irregularity and defects of the myosepta, whereas immunofluorescence with an anti-MHC antibody demonstrated myofibril injury (Fig. 2). These structural changes in the skeletal muscle after 25B-NBOMe-treatment were consistent with rhabdomyolysis.

5-HT2A receptor antagonists prevented 25B-NBOMe-induced hypo-locomotion, death and muscle injury

Zebrafish larvae were co-treated with 25B-NBOMe (0.5 μg/mL) and a 5-HT subtype inhibitor for 2 days. The 5-HT2A receptor antagonists, aripiprazole and ritanserin, significantly improved the survival rate of the 25B-NBOMe-treated larvae (*p = 0.032 and **p = 0.013, respectively). However, the 5-HT1A + 5-HT1B antagonist (β-AR blocker) propranolol, and the 5-HT3 receptor antagonist granisetron did not affect the survival of the 25B-NBOMe-treated larvae (Fig. 3a). The increase in the ratio of larvae with reduced birefringence by 25B-NBOMe-treatment were significantly reduced by aripiprazole or ritanserin (*p = 0.023 and **p = 0.013, respectively) (Fig. 3b). These findings indicated that 5-HT2A receptor was involved in rhabdomyolysis induced by 25B-NBOMe. Behavioral analysis demonstrated that ritanserin, but not aripiprazole or granisetron, significantly reduced 25B-NBOMe-induced hypo-locomotion (reduced swimming distance) (*p = 0.023) (Fig. 3c). On the other hand, propranolol enhanced the 25B-NBOMe-induced hypo-locomotion (**p = 0.020). Expression of the 5-HT2A receptor in zebrafish skeletal muscle was confirmed by reverse transcription polymerase chain reaction (Fig. 3d).

Discussion

In zebrafish larvae, 25B-NBOMe, one of the most potent 5-HT2A agonists known to date, induced lethal rhabdomyolysis (Fig. 1a). The rhabdomyolysis was confirmed not only by the reduction in muscle birefringence (Fig. 1c), but also by the reduced immunostaining for a sarcolemmal (myoseptal) protein (β-dystroglycan) and myofibrillar protein in skeletal muscle (Fig. 2). The 25B-NBOMe-induced rhabdomyolysis was prevented by treatment with either...
aripiprazole or ritanserin (5-HT\textsubscript{2A} antagonists), but not by propranolol (5-HT\textsubscript{1A} + 5-HT\textsubscript{1B} antagonist) or granisetron (5-HT\textsubscript{3} antagonist). These findings confirmed the induction of 5-HT\textsubscript{2A}-dependent rhabdomyolysis by 25B-NBOMe-treatment. However, according to a review on 5-HT receptors [19], the 5-HT\textsubscript{2A} receptor is implicated in the contraction of smooth muscle, but the presence of 5-HT\textsubscript{2A} receptors in skeletal muscle was not mentioned.

In the skeletal muscle of young and adult rats, 5-HT\textsubscript{2A} receptors were shown to localize to the sarcolemma and T-tubules, respectively [21]. In zebrafish muscle, however, the localization of 5-HT\textsubscript{2A} could not be analyzed, because there were no anti-5-HT\textsubscript{2A} antibodies available with reactivity to the zebrafish epitope. Instead, we could confirm the presence of a 5-HT\textsubscript{2A}-receptor gene in the zebrafish (Fig. 3d).

In rodent skeletal muscle, it was shown that 5-HT\textsubscript{2A} activation contributed to muscle differentiation and glycolysis. Via 5-HT\textsubscript{2A}, 5-HT induced the transcriptional activation of myogenin and glucose transporter 3, thereby promoting muscle differentiation and glycolysis, respectively [22]. Additionally, 5-HT was shown to activate the key glycolytic enzyme 6-phosphofructo-1-kinase [23]. The activation of glycolysis can enhance muscle contraction via an increase in intracellular adenosine triphosphate (ATP) and Ca\textsuperscript{2+} levels. In cardiomyogenic cells cultured in a high glucose medium, we demonstrated that hypoxia induces excessive glycolysis accompanied by metabolic acidosis (excessive intracellular H\textsuperscript{+}), an increase in intracellular Na\textsuperscript{+} via the Na\textsuperscript{+}/H\textsuperscript{+}-exchanger, an increase in intracellular Ca\textsuperscript{2+} via the Na\textsuperscript{+}/Ca\textsuperscript{2+}-exchanger, and finally cell death via the Ca\textsuperscript{2+}-dependent protease calpain [24]. It remains to be clarified as to whether 25B-NBOMe causes an over-activation of glycolysis and increases intracellular ATP and Ca\textsuperscript{2+} levels, resulting in rhabdomyolysis.

Muscle hypertonicity and hyperthermia are predominant manifestations of serotonin syndrome, reflecting rhabdomyolysis in general [2], which is induced by 25B-NBOMe [7, 8]. Additionally, a few studies have suggested that 5-HT\textsubscript{2A} stimulation enhances muscle contraction under particular conditions. In spinal cord injury, persistent inward Ca\textsuperscript{2+} currents induce muscle spasms via the activation of 5-HT\textsubscript{2} and \(\alpha\)-adrenergic receptors [25], which can be activated also by 25B-NBOMe [8]. In excitable cells, 5-HT and the serotonergic drug MDMA modulates Ca\textsuperscript{2+}-driven signals through the coupling of L-type Ca\textsuperscript{2+}-channels and serotonin transporters [26]. Given its potent 5-HT\textsubscript{2A} agonistic effects [5], 25B-NBOMe may induce intracellular Ca\textsuperscript{2+} overload and skeletal muscle over-contraction, in association with rhabdomyolysis. The latter possibility remains to be addressed.

Rhabdomyolysis occurs not only in serotonin syndrome, but also in malignant hyperthermia (MH). MH is characterized by severe hyperthermia and rhabdomyolysis via excessive sarcoplasmic reticulum Ca\textsuperscript{2+} release [27]. Similarly to anesthetics, the 5-HT\textsubscript{2A} agonist 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride induced rapid and intense contraction in muscle isolated from MH patients, compared with that from healthy volunteers [28], and the hyper-contraction was prevented by ritanserin [27]. In the well-established rat model of serotonin syndrome,
co-administration of 5-hydroxy-L-tryptophan (the 5-HT precursor) and clorgyline (a MAO inhibitor) induced lethal hyperthermia, which was prevented by 5-HT2A antagonists (ritanserin and pipamperone), but not by the 5-HT1A + 5-HT1B antagonist propranolol [29], as we found in this zebrafish model of rhabdomyolysis induced by 25B-NBOMe (Fig. 3a–c). On the other hand, fluoxetine (an SSRI) suppresses impaired muscle birefringence in a zebrafish model of Duchenne muscular dystrophy, suggesting the implication of serotonin reuptake in dystrophic muscle [16]. Collectively, we hypothesized that excessive serotonin uptake and 5-HT2A-activation may generally induce muscle degeneration including rhabdomyolysis.

Stewart et al. [10] proposed that zebrafish provide a promising model to investigate serotonin syndrome due to genetic homology to humans, genetic tractability, and low cost of maintenance. Our zebrafish model of rhabdomyolysis with 25B-NBOMe has many advantages over the frequently utilized rat model. In the rat model, rhabdomyolysis can only be analyzed by body temperature and creatine kinase release into the blood. In the zebrafish model, however, we can directly observe and analyze the temporal progression of muscle degeneration by the birefringence assay due to their transparency. Additionally, whole-body immunofluorescence for myosepta and myofibril proteins visualized the disruption of the two structures by a simple procedure. Moreover, due to the small sizes and large numbers of available fish, high-throughput analyses can be performed under diverse conditions in a short period of time, at low costs, with minimal effort, and with small amounts of drugs and fewer animals.

In zebrafish, various behavioral parameters of psychiatric disorders, including those due to drug abuse, can be temporally recorded for high-throughput, unbiased and semi-quantitative analyses using video-tracking technologies [10]. Stewart et al. [10] proposed that surface dwelling and hypo-locomotion in zebrafish after the administration of serotoninergic drugs represented serotonin syndrome. Consistently, we confirmed 25B-NBOMe-induced hypo-locomotion in our zebrafish larva model. This hypo-locomotion was prevented by the 5-HT2A antagonist ritanserin, but not by the 5-HT2A antagonist aripiprazole (Fig. 3c). Given that the two 5-HT2A antagonists prevented the 25B-NBOMe-induced reduction in muscle birefringence to a similar extent (Fig. 3b), this indicates that muscle weakness does not induce hypo-locomotion. Differences in the effects of the two 5-HT2A antagonists on 25B-NBOMe-induced hypo-locomotion may be due to the fact that ritanserin, but not aripiprazole, can inhibit 5-HT3C and 5-HT5 receptors which affect locomotion [19]. On the other hand, Wappler et al. [30] proposed that serotonin syndrome is induced by overstimulation of the central nervous system 5-HT1A receptor by high synaptic 5-HT levels, in association with its interaction with 5-HT2A and dopaminergic receptors. However, the non-specific 5-HT1A antagonist propranolol did not prevent, but rather aggravated the 25B-NBOMe-induced hypo-locomotion (Fig. 3c). Therefore, although it has been believed that rhabdomyolysis is one of the diverse symptoms of serotonin syndrome, our study suggests that rhabdomyolysis and behavioral manifestations of serotonin syndrome are mediated by different 5-HT receptor subtypes and pathways.

Conclusions

In recent years, zebrafish have been emerging as a useful animal model in various fields of the neurosciences, including psychology, pharmacology, toxicology and even psychiatry, because the small fish have various advantages such as their high homology to humans, genetic tractability, low cost and high-throughput analyses. In this study, we presented a simple and reproducible model of rhabdomyolysis induced by 25B-NBOMe, a potent hallucinogenic designer drug, using zebrafish larvae, which indicated that the 5-HT2A receptor was involved in the formation of rhabdomyolysis. The zebrafish have a high potential to be utilized for assessing the pharmacological, neurobehavioral and toxic efforts of novel psychotropic substances, and for studies on the mechanisms of their effect manifestation in forensic toxicology.

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Compliance with ethical standards

Conflict of interest There are no financial or other relations that could lead to a conflict of interest.

Ethical approval The use of zebrafish for this study was approved by the Experimental Animal Committee of Tokyo Medical University (approval number: S28029). This article does not contain any studies with human participants performed by any of the authors.

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