Overexpression of Human MRP1 in Neurons causes resistance to Antiepileptic Drugs in Drosophila Seizure Mutants

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Abstract: Multidrug resistance-associated protein 1 (MRP1), an efflux multidrug transporter, was shown to be elevated in both glia and neurons in seizure focus in refractory epilepsy patients. Up-regulation of MRP1 and other multidrug transporters in perivascular astrocytes was suggested to cause resistance to antiepileptic drugs (AEDs) by reducing the concentration of AEDs at the epileptogenic areas. However, it is not known whether the up-regulation of MRP1 in neurons can cause resistance to AEDs, such as sodium phenytoin (PHT) and valproic acid (VPA). PHT inhibits voltage-gated sodium channel (VGSC) by occluding it, but whether PHT enters the channel through its inner or outer pore is not known. The authors overexpressed human MRP1 protein only in neurons in a Drosophila genetic seizure model, bang senseless (bss) mutants. The authors found that overexpression of MRP1 blocked the attenuation of the seizure behavior of bss mutants by acute and chronic application of PHT, and by chronic application of VPA. Conversely, overexpression of MRP1 in neurons increased the tolerance of bss flies to high-dosage PHT and VPA. Thus, up-regulation of MRP1 expression only in neurons causes resistance to AED in seizure flies. Moreover, the current data suggest that PHT enters VGSC through its inner pore.

Keywords: refractory epilepsy (RE), bang senseless (bss), transporter, voltage-gated sodium channel (VGSC), pore, sodium phenytoin (PHT), valproic acid (VPA)

INTRODUCTION

Epilepsy is one of the most common neurological disorders, the majority of which can be controlled effectively by antiepileptic drugs (AEDs). However, the seizure in about one third of epileptic patients is resistant to a broad range of AEDs (Sander, 1993), and the epilepsy in this group of cases is considered to be refractory epilepsy (RE). For a comprehensive definition of drug resistant epilepsy, please refer to a special report (Kwan et al., 2010).

P-glycoprotein (P-gp) and some multidrug resistance associated proteins (MRPs) can transport some anticancer drugs out of cells against concentration gradient (Borst & Schinkel, 1997), and cause cellular resistance to anticancer drugs. P-gp and some MRPs were also reported to transport some AEDs out of cells (Loscher & Potschka, 2002). In the epileptogenic areas in RE, the expression of P-gp and some MRPs was found to be up-regulated in capillary endothelial cells and perivascular astrocytes (Kwan & Brodie, 2005; Loscher & Potschka, 2002; Sisodiya, 2007). The overexpression of multidrug transporters may facilitate the transport of AEDs from the extraneuronal space back to the blood, and produce an inadequate extracellular drug concentration in the epileptogenic areas. Therefore, overexpression of multidrug transporters at epileptogenic locus was suggested to cause resistance to AEDs in RE (Kwan & Brodie, 2005; Loscher & Potschka, 2002; Sisodiya, 2007). Consistently, inhibition of P-gp and/or MRPs by drugs and knockout of their corresponding genes increased the extracellular concentration of some AEDs in the rodents (Kwan & Brodie, 2005).
However, P-gp, but not three of the MRPs (MRP1, MRP2, and MRP5), was confirmed to be an efflux transporter of some AEDs, including phenytoin, carbamazepine, valproate, levetiracetam, lamotrigine, and phenobarbital in a highly sensitive assay (Luna-Tortos et al., 2008, 2010). Moreover, MRP1 was more frequently found to be overexpressed in neurons than in glial cells in focal cortical dysplasia (FCD) and ganglioglioma (GG) (Aronica et al., 2003), which are two major causes of pediatric refractory epilepsy. Whether the overexpression of MRP1 in neurons contributes to AEDs resistance in RE is not clear.

Drosophila melanogaster bang-sensitive paralytic mutants have been frequently used as seizure models for identification of genes important in epilepsy, and analysis of seizure susceptibility and AED action (Kuebler & Tanouye, 2002; Lee & Wu, 2002; Reynolds et al., 2004; Song et al., 2007). bang senseless (bss) mutants are such bang-sensitive paralytic mutants. bss mutants exhibit typical hyperactive behavior and paralysis, following a mechanical shock, such as a tap of the culture vial on the bench or a brief vortex mixing. The hyperactivity is characterized by intense, uncoordinated motor activities, including wing flapping, leg shaking, and abdominal muscle contracting, which are comparable to the mammalian seizure behavior (Benzer, 1971). Moreover, the hyperactive behavior and paralysis are associated with a concomitant electrical seizure activity and failure in a neural circuit (Pavlidis & Tanouye, 1995). Acute and chronic application of some AEDs, such as phenytoin (PHT), can significantly reduce the duration of hyperactive behavior and paralysis of bss flies (Kuebler & Tanouye, 2002; Reynolds et al., 2004). PHT is known to inhibit voltage-gated sodium channel (VGSC) by binding to a site inside the channel in a voltage- and frequency-dependent manner (Mantegazza et al., 2010; Ragsdale et al., 1996). However, whether PHT reaches the binding site from the extracellular or intracellular side of the VGSC is not known.

In the present study, we investigated the effect of pure neuronal overexpression of MRP1 protein on the amelioration of hyperactive behavior and paralysis of bss flies by PHT and another common AED—valproic acid (VPA), and on the tolerance of bss flies to high-dosage of the two AEDs.

MATERIALS AND METHODS

Stocks and Genetics

Drosophila stocks were cultured on standard medium and entrained into 12-hour light/dark cycle at 23°C to 25°C. The virgins of bang senseless1 (bss1) (Ganetzky & Wu, 1982; Lee & Wu, 2002) mutants were crossed to each of w1118, elav-Gal4 (P[Gal4-elav.L]3), UAS-MRP1, and UAS-MRP1; elav-Gal4 transgenic flies to generate groups of control and subject male flies. The details for generating the UAS-MRP1 transgenic line is below.

Vector Construction and Generation of UAS-MRP1 Transgenic Fly

pUAST-MRP1 plasmid was constructed by cloning the Eagl1/Kpn1 fragment from pcDNA3.1(−)-MRP1 vector (Ito et al., 2001), which contains the full coding sequence of human MRPI, into pUAST plasmid digested with Eagl1/Kpn1enzymes. The resulting pUAST-MRP1 plasmid was sequenced and validated. Plasmid pUAST-MRP1 was then transformed into w1118 embryos to produce UAS-MRP1 transgenic lines. The transgenic lines were validated by polymerase chain reaction (PCR).

Drug Treatment and Behavioral Testing

PHT (sodium diphenylhydantoin; Sigma, St. Louis, MO, USA) and VPA (valproic acid sodium; Sigma, St. Louis, MO, USA) were chosen for the experiment according to a previous study with Drosophila (Reynolds et al., 2004). Both PHT and VPA are water soluble, thus we dissolve them in water by vortexing and heating. For acute exposure to PHT, 15 flies at the age of 1–3 days from each of subject and control groups were transferred into each vial containing filter paper coated with 1% sugar solution, which contains PHT or VPA at a specific concentration (0, 0.05, 0.1, or 1.0 mg/mL). After feeding on the drug-containing filter paper for 24 hours, flies were subjected to behavioral testing. For chronic exposure to PHT, PHT was mixed with Blue Instant Medium (Carolina Biological Supply, Burlington, NC) with desired concentrations (0, 0.1, or 1.0 mg/mL), and then placed into culture vials. Ten pairs of parent flies for generating subject and control flies placed into these vials and their progeny were raised on the drug-containing food. Three to 5 days after eclosion, the desired subject and control flies were selected from the progeny.

Vortexing Assay

Testing for bang-sensitive paralysis was performed on flies at the age of 3–5 days post eclosion. Flies were rested for a minimum of 2 hours following CO₂ anesthesia prior to testing. Approximately 15 flies were then placed into a clean vial and allowed to rest for an additional 30 minutes. These flies were vortexed on VORTEX-5 (Kylin-Bell Lab Instruments, Haimen, China) at maximum speed for 10 seconds. Mostly, all flies displayed a period of hyperactivity followed by paralysis. The whole process was recorded.
with video camera. Flies were considered to be recovered when they were able to resume an upright standing position. Mean recovery time (MRT) was calculated from the cease of vortexing to flies’ recovery.

Eclosion Rate Assays

In order to observe the impact of drugs on the Drosophila growth and development, 300 embryos of each of subject and control groups were transferred 100 per vial to fresh PHT- or VPA-containing food at the concentrations, 0, 0.1, or 1.0 mg/mL. After 12–14 days, young flies were harvested by CO2 exposure, sorted by sex, and the eclosion rate was determined.

Data Analysis and Statistics

SPSS (SPSS, Chicago, IL, USA) software was used for data analysis. If not described elsewhere, one-way analysis of variance (ANOVA) followed by Fisher’s least significance difference (LSD) was employed to analyze statistical significance. Statistical significance was defined as \( p < 0.05 \). The data were presented as mean ± SD if not specified otherwise.

RESULTS

The Effect of MRP1 Overexpression on Acute Application of PHT and VPA

To test the effect of MRP1 overexpression on the therapeutic effect of acute PHT and VPA treatment, male \( bss^{1} \) mutant flies with (subject flies) or without (control flies, three groups) pan-neuronal overexpression of human MRP1 were fed with PHT or VPA solution at the concentrations of 0, 0.05, 0.1, and 1.0 mg/mL for 24 hours. The genotypes of subject and control groups are listed in Table 1. Feeding with PHT of 0.05 and 0.1 mg/mL did not cause obvious lethality in either subject or control groups, but feeding with PHT of 1.0 mg/mL killed the majority of the control groups and relatively less subject group (data not shown). Feeding with VPA of 0.05, 0.1, and 1.0 mg/mL did not cause obvious lethality in either subject or control groups. After exposure to PHT or VPA, subject and control groups were simultaneously stimulated by a vortex mixing, their hyperactive behavior and paralysis were recorded and their mean recovery time (MRT) from seizure and paralysis were measured.

In the three control groups containing \( bss^{1} \) mutation, acute application of PHT at the concentrations of 0.05 and 0.1 mg/mL significantly reduced the MRTs in a dosage-dependent manner (Figure 1). However, acute treatment with PHT of 1.0 mg/mL did not change the MRTs (Figure 1), it might be due to the toxicity of high-dosage PTH. In contrast, in the corresponding subject group, acute application of PHT at the concentration of 0.05 or 0.1 mg/mL did not significantly change the MRT, but acute treatment with PHT of 1.0 mg/mL significantly reduced the MRT (Figure 1). In both subject and control groups containing \( bss^{1} \) mutation, acute application of VPA at the concentrations of 0.05, 0.1, and 1.0 mg/mL did not cause detectable significant change in the MRTs (data not shown). Thus, in the subject group containing \( bss^{1} \) mutation and its three control groups, acute PHT but not VPA treatment significantly ameliorated the seizure behavior.

Table 1. The group assignment and genotype of flies.

<table>
<thead>
<tr>
<th>Group assignment for flies</th>
<th>Genotype of groups with ( bss^{1} )</th>
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<tbody>
<tr>
<td>Subject</td>
<td>( bss^{1} ); ( \text{UAS-MRP1}^{+/+} ); ( \text{elav-Gal4}^{+/+} )</td>
</tr>
<tr>
<td>Control 1</td>
<td>( bss^{1} )</td>
</tr>
<tr>
<td>Control 2</td>
<td>( bss^{1} ); ( \text{elav-Gal4}^{+/+} )</td>
</tr>
<tr>
<td>Control 3</td>
<td>( bss^{1} ); ( \text{UAS-MRP1}^{+/+} )</td>
</tr>
</tbody>
</table>

Note. The subject group consists of male \( bss^{1} \) flies containing the \( \text{UAS-MRP1} \) transgene and expressing the \( \text{elav-Gal4} \) transcription factor. \( \text{Elav-Gal4} \) drives the expression of \( \text{UAS-MRP1} \) transgene in all neurons in subject flies. Control 2 consists of male \( bss^{1} \) flies expressing only \( \text{elav-Gal4} \) transcription factor, whereas control 3 consists of male \( bss^{1} \) flies containing only \( \text{UAS-MRP1} \).

Figure 1. Overexpression of MRP1 in neurons blocks the amelioration of seizure behavior of \( bss^{1} \) flies by acute exposure to PHT. The effect of acute PHT application on the MRTs in control and subject groups. For each column, \( 33 \leq n \leq 40 \). *\( p < 0.05 \), **\( p < 0.01 \), and ***\( p < 0.001 \), respectively, when compared with the MRT of the same group without PHT treatment. *\( p < 0.05 \), **\( p < 0.01 \), and ***\( p < 0.001 \), respectively, when compared with the MRTs of three control groups treated with PHT at the same concentration.
and overexpression of MRP1 reduced the therapeutic effect of the acute treatment of PHT.

The Effect of MRP1 Overexpression on Chronic Treatment of PHT and VPA

To test if pan-neuronal overexpression of human MRP1 reduces the “therapeutic” effect of chronic application of PHT and VPA on bss\(^1\)-containing animals, we raised subject and control groups in culture medium containing PHT or VPA of 0, 0.1, or 1.0 mg/mL since they were at the embryonic stage. After chronic exposure to PHT or VPA, the eclosion rates and MRTs of subject and control groups were examined.

In the animals containing bss\(^1\) mutation, when the concentration of PHT and VPA was 0.1 mg/mL, the eclosion rate of each group was not markedly changed, and the eclosion rate of subject group was not significantly different from those of control groups (Figure 2A). In contrast, when the concentration of PHT and VPA was 1.0 mg/mL, the eclosion rate of each group was markedly reduced, but the eclosion rate of subject group was dramatically higher than those of control groups (Figure 2A). These results demonstrate that chronic treatment with high-dosage PHT or VPA caused toxicity in animals containing bss\(^1\) mutation, and that overexpression of human MRP1 in the neurons rendered bss\(^1\)-containing animals resistant to the toxicity of both chronic high-dosage PHT and VPA treatment.

We also raised Elav-containing wild-type animals with and without MRP1 overexpression in culture medium containing PHT or VPA of 0, 0.1, or 1.0 mg/mL since they were at the embryonic stage. After chronic exposure to PHT or VPA, the male eclosion rates of both groups were examined. As in the animals containing bss\(^1\) mutation, the eclosion rate of each group was not obviously changed when the concentration of PHT and VPA was 0.1 mg/mL, but was markedly reduced when the concentration of PHT and VPA was 1.0 mg/mL (Figure 2B). Moreover, the eclosion rate in male wild-type animals with MRP1 overexpression was significantly higher than that in male wild-type animals without MRP1 overexpression when the concentration of PHT was 1.0 mg/mL (Figure 2B, left).

Three to 5 days after eclosion, vortexing assay was conducted on the flies with chronic treatment of 0.1 mg/mL PHT or VPA, and on the flies with 0 mg/mL drug treatment. In the control groups containing bss\(^1\) mutation, the MRTs were significantly reduced by both PHT

![Figure 2. The effect of MRP1 overexpression in neurons on the eclosion rate of w\(^{1118}\) and bss\(^1\) mutants with chronic exposure to PHT or VPA. (A) The eclosion rates of male subject and control animals containing bss\(^1\) mutation cultured with PHT or VPA at specific concentrations. (B) The eclosion rates of male Elav-containing wildtype animals with and without MRP1 overexpression cultured with PHT or VPA at specific concentrations. The data represent the results from triplicate experiments. *p < 0.05; **p < 0.01.](https://www.jneurogenet.com/10.1007/s10983-009-9235-8)
and VPA treatments (Figure 3). However, in the subject groups containing bss mutant, the MRTs were not significantly changed by PHT treatment (Figure 3), or even significantly increased by VPA treatment (Figure 3). In Elav-containing wild-type flies with and without MRPl overexpression, vortexing never caused paralysis (data not shown). These results demonstrate that chronic exposure to PHT or VPA at the concentration of 0.1 mg/mL ameliorated the seizure behavior of bss-containing animals, and overexpression of human MRPl in the neurons abolished this therapeutic effect.

Both PHT and VPA had negative effect on eclosion rate but positive effect on seizure behavior. Chronic application of each drug at 0.1 mg/mL did not obviously change the eclosion rate, but significantly reduced the MRT of bss flies, indicating that the concentration for each drug to produce negative effect on eclosion was higher than that to inhibit seizure behavior.

**DISCUSSION**

In bss and some other seizure Drosophila models, MRT correlates very well with the seizure activities of neurons. Moreover, behavioral seizure activity and frequency are the major criteria for evaluating the therapeutic efficacy of AEDs in clinic. Thus, it is appropriate to examine the effect of AEDs on seizure activity in bss mutants by measuring MRT. Overexpression of human MRPl in neurons with a neuron specific driver prevented the “therapeutic” effect of PHT and VPA on the seizure activity of bss mutant flies, and produced resistance to the lethal effect of high dosage PHT and VPA in bss mutant flies. Without drug treatment, the MRTs of both subject flies were indistinguishable from those of control flies, indicating the MRPl overexpression did not change the neuronal excitability or homeostasis. These results provide direct in vivo evidence showing the cause-and-effect relationship between neuronal overexpression of human MRPl and the resistance to PHT in seizure animals.

In contrast to the effect of acute PHT treatment, acute treatment with VPA did not ameliorate the seizure behavior of bss mutant flies. We do not know the exact reason, but we speculate that the absorbing, transporting, and accumulating of VPA may be slower than those of PHT in Drosophila, therefore the concentration of VPA could not reach the level to suppress seizure behavior within 24 hours. Consistently, acute treatment with 1.0 mg/mL PHT but not VPA killed the majority of animals in all groups.

The antiepileptic effect of PHT is mainly mediated by its inhibition of voltage-gated sodium channel (VGSC). PHT is known to inhibit VGSC by binding to a site inside the channel in a voltage- and frequency-dependent manner (Mantegazza et al., 2010; Ragsdale et al., 1996). However, whether PHT reaches the binding site from the extracellular or intracellular pore of the channel is not known. Given that human MRPl is an efflux transporter, the amelioration of antiepileptic effect of PHT by the neuronal overexpression of MRPl was presumably mediated by preventing the intraneuronal accumulation of PHT, and in turn blocking the binding of PHT to VGSC. If this was the case, our data suggest that PHT may get into VGSC through its inner pore. To determine this, the time course and efficacy of the inhibition of VGSC by PHT in “outside-out” and “inside-out” patch-clamp configurations need to be investigated.

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REFERENCES


