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# Short-term exposure to L-type calcium channel blocker, verapamil, alters the expression pattern of calcium-binding proteins in the brain of goldfish, *Carassius auratus*

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ABSTRACT

The influx of calcium ions ($Ca^{2+}$) is responsible for various physiological events including neurotransmitter release and synaptic modulation. The L-type voltage dependent calcium channels (L-type VDCCs) transport $Ca^{2+}$ across the membrane. Calcium-binding proteins (CaBPs) bind free cytosolic $Ca^{2+}$ and prevent excitotoxicity caused by sudden increase in cytoplasmic $Ca^{2+}$. The present study was aimed to understand the regulation of expression of neuronal CaBPs, namely, calretinin (CR) and parvalbumin (PV) following blockade of L-type VDCCs in the CNS of *Carassius auratus*. Verapamil (VRP), a potent L-type VDCC blocker, selectively blocks $Ca^{2+}$ entry at the plasma membrane level. VRP present in the aquatic environment at a very low residual concentration has shown ecotoxicological effects on aquatic animals. Following acute exposure for 96 h, median lethal concentration ($LC_{50}$) for VRP was found to be 1.22 mg/L for goldfish. At various doses of VRP, the behavioral alterations were observed in the form of respiratory difficulty and loss of body balance confirming the cardiovascular toxicity caused by VRP at higher doses. In addition to affecting the cardiovascular system, VRP also showed effects on the nervous system in the form of altered expression of PV. When compared with controls, the pattern of CR expression did not show any variations, while PV expression showed significant alterations in few neuronal populations such as the pretectal nucleus, inferior lobes, and the rostral corpus cerebellum. Our result suggests possible regulatory effect of calcium channel blockers on the expression of PV.



## 1. Introduction

The pharmaceutical drug verapamil (VRP) is a potent L-type calcium channel blocker of phenylalkylamine category and is widely used for treating angina pectoris, cardiac arrhythmia, coronary heart diseases, hypertension, and cluster headaches (Beck et al., 2005; Godfraind et al., 1986; Opie, 1982). VRP blocks calcium influx into cardiac myocytes of the cardiac conduction system and smooth muscles of the vascular system, thereby causing reduced myocardial contractility, prolonged conduction time, and vascular relaxation (Batalis et al., 2007). VRP shows high-affinity toward the inactivated state of L-type voltage dependent calcium channels (L-type VDCCs), which is responsible for decreasing the rate of channel conformational change back from the inactivated to the resting or open states, and so stabilizes the inactivated state of these channels (Hockerman et al., 1997). L-type VDCCs are responsible for influx of $Ca^{2+}$ into the cytosol of neurons, thereby increasing their intracellular concentration (Voglis and Tavernarakis, 2006) and antagonists like VRP affect this influx through these channels (Yamakage and Namiki, 2002). VRP blocks entry of $Ca^{2+}$ at the level of plasma membrane and is a slow calcium channel blocker (Godfraind et al., 1986). In addition to cardiac myocytes and smooth muscles, VRP also acts on neuronal calcium channels (Ito et al., 1984). Various types of VDCCs occur in the nervous system (Cao, 2006) and among these, L-type calcium channels are the most efficient ones in regulating activity dependent gene expression. VRP has been used extensively as a drug in human patients, and its effect has been elaborately reported in case of mammals as well (Karmansky and Gruener, 1997; Pinar et al., 1998). VRP, when administered orally, undergoes oxidative metabolism mediated by a category of cytochrome P450 enzymes (Busse et al., 1995; Kroemer et al., 1993). P-glycoprotein acts as an efflux pump that restricts the accumulation of foreign chemicals like pharmaceuticals (Kim et al., 1998) and therefore helps in the elimination of these chemicals. VRP itself (Tsuruo et al., 1982) and few of its metabolites (Pauli-Magnus et al., 2000) inhibit the activity of P-glycoprotein. Inhibition of P-glycoprotein increases the bioavailability of these orally administered pharmaceutical drugs (Mayer et al., 1996). Therefore, it is evident that in addition to treating arrhythmia, VRP affects other cellular functions as well. VRP affects the nervous system by impairing long-term memory and plasticity in rats (Seoane et al., 2009). After metabolism, approximately 3–4 % VRP is excreted in urine in the unchanged form (Eichelbaum et al., 1979; Mikus et al., 1990). Furthermore, residual concentrations (0.058–0.9 μg/L) of VRP have been reported from sewage

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and sewage treatment plants in Australia contaminating the groundwater (Al-Rifai et al., 2007; Khan and Ongerth, 2004).

VRP affects the cardiovascular system and the overall morphology in fish (Steinbach et al., 2013). VRP leads to reduced heart rate in fishes (Berghmans et al., 2008; Shin et al., 2010; Steinbach et al., 2013) similar to that in humans (Grossman and Messerli, 2004). The similar effects of VRP on fish and human cardiovascular system can be justified because the polypeptide sequences of the α-subunit of the L-type calcium channel, which is the target of VRP, are 78% similar in humans and zebrafish (Lacinová et al., 1995; Rottbauer et al., 2001). Furthermore, according to studies performed on common carp embryos and larvae, VRP causes morphological deformities, such as spine malformations, edemas, and notable hematocele in the intestine (Steinbach et al., 2013).

Recently, behavioral analysis has gained importance as a valuable parameter for ecotoxicological studies on aquatic animals (Melvin and Wilson, 2013). The effects of acute and chronic VRP exposure on various morphological, behavioral, hematological parameters and antioxidant systems in the nervous system of rainbow trout have been reported (Li et al., 2010; Li et al., 2011a). Although, in mammals a certain class of cytochrome P enzymes metabolizes VRP, some of these enzymes do not alter the activity of VRP in teleosts (Burkina et al., 2012). Hence, it is evident that the metabolism and action of VRP could be different across various vertebrate classes.

The calcium antagonists like VRP block $Ca^{2+}$ influx and may thereby modulate expression and activity of intracellular proteins. The cytoplasmic calcium-binding proteins (CaBPs) are the group of proteins that bind free $Ca^{2+}$ and prevent cell death due to excitotoxicity. CaBPs like calretinin (CR) and parvalbumin (PV) are used as neuroanatomical markers, as only specific subsets of neuronal populations express these proteins (Andressen et al., 1993). However, the information about their regulation and involvement in various functional pathways in the nervous system of teleosts is scanty. Fish exposed to acute exposure of experimental doses of VRP showed altered behavioral patterns (Li et al., 2010). However, there is no report on the effects of VRP on the expression patterns of CR and PV in CNS. Therefore, in the present study, the goldfish, *Carassius auratus*, were exposed to various doses of VRP to understand probable regulation of CR and PV expression following blockade of $Ca^{2+}$ influx through L-type VDCCs by the action of VRP. The behavioral patterns of exposed goldfish were used as indicators for acute VRP toxicity, as reported in rainbow trout (Li et al., 2010).

## 2. Materials and methods

### 2.1. Animals

The goldfish, *Carassius auratus* (30–70 mm in length), irrespective of sex, were procured from a local dealer and brought to the laboratory. The animals were in healthy condition and devoid of any apparent malformations. The fish were allowed to recover from the stress and acclimatize to the laboratory conditions of 25 ± 3 °C and 12 h dark/12 h light cycle. These laboratory conditions were maintained throughout the period of experimentation. A commercial fish food was fed to the fish twice a day during acclimatization phase until 24 h prior to the VRP treatment. At the beginning of the experiment, all the animals were randomly separated in different experimental groups. During the entire period of VRP exposure, animals were not fed to avoid any effect of food on the experimental outcomes.

### 2.2. Experimental design and VRP doses

Acute toxicity of VRP test was carried out in accordance with the OECD guideline for testing of chemicals No. 203 "Fish, Acute Toxicity Test" (OECD, 1992). For VRP exposure, fish were distributed in seven groups of 14 animals each. Six experimental concentrations of VRP, such as 0.25, 0.5, 1.0, 2.0, 5.0, 10.0 mg/L, were used for groups I–VI, respectively. A simultaneous control group was maintained with no VRP added in the aquarium water keeping all the other conditions constant with those of experimental ones. The drug (±)-verapamil.hydrochloride (Enzo Life Sciences, Farmingdale, New York, USA) was dissolved in distilled water to prepare 50 mg/ml stock solution. Fresh stock solutions were prepared every day just prior to use.

For acute VRP treatment, the semi-static system was used, where the test solution was renewed after every 24 h to maintain the drug concentration. Mortality in fish groups was checked frequently. The dead fish were removed immediately and discarded. The appropriate safety and disposal procedures were followed. During the entire period of experiment, fish behavior was observed for any anomalies. All the experimental procedures were carried out in accordance with the ethical committee guidelines of the RTM Nagpur University, Nagpur, India.

### 2.3. Statistical analysis

Total mortality after 96 h was counted and percent mortality was calculated. These values were considered for calculating median lethal concentration ($LC_{50}$) using StatsDirect Statistical Software Version 2.8.0 based on Finney's Probit Analysis Method (Finney, 1971).

### 2.4. Tissue processing

The animals that survived the test period only were anaesthetized using paraldehyde (3 ml/L). However, as all the fish were dead in group VI, where the drug concentration (10 mg/L) was the maximum, the tissues were procured from these fish as soon as they were dead. The animals were decapitated immediately and the heads with the lower jaw removed were fixed in Bouin's fixative for 24 h at 4 °C. The heads were then cryoprotected using 30% sucrose and frozen embedded in 15% polyvinylpyrrolidone. The brain blocks were cut in transverse plane at a thickness of 15 μm on Cryotome E (Thermo Scientific, UK). The sections were mounted on poly-L-lysine coated slides for immunostaining.

### 2.5. Immunohistochemistry

The cryosections were rinsed in Tris-buffered saline (TBS) (0.1 M, pH 7.4). The slides were immersed in a solution of 0.3% Triton X-100 and 1% $H_2O_2$ in TBS for 30 min to eliminate the activity of endogenous peroxidase in the tissues, followed by TBS washes. Non-specific binding of the antibody was prevented by a 60 min treatment with 1% BSA in TBS. The sections were then incubated with a solution of either polyclonal rabbit antibody against calretinin (CR) (Swant, Bellinzona, Switzerland) at 1:1000 dilutions or monoclonal mouse antibody against parvalbumin (PV) (Swant, Bellinzona, Switzerland) at 1:2000 dilutions in TBS at room temperature (RT) for 18 h. Then the sections were rinsed in TBS and incubated for 1 h at RT with rabbit IgG and mouse IgG raised in goat (1:200) (Vector Laboratories Inc., Burlingame, CA, USA) against CR and PV antibodies, respectively. Subsequently, the sections were rinsed in TBS and incubated for 1 h with ABC reagent (Vector Laboratories Inc., Burlingame, CA, USA), followed by TBS washes. The peroxidase reaction was then developed and visualized by incubating sections in a solution of TBS containing 0.06% 3, 3′-diaminobenzidine (DAB) (Amresco Inc, OH, USA) as a chromogen and 0.05% $H_2O_2$ as a substrate for 10–20 min. Finally, the sections were dehydrated in ethanol, cleared in xylene, and mounted in DPX. Negative control reactions were performed for checking the specificity of antibodies by omitting the primary and secondary antibodies. No positive immunoreactions were observed in these control reactions.

### 2.6. Structural analysis

For identifying various nuclei of the goldfish brain, various articles illustrating fish brain cytoarchitecture were referred (Braford and