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THE HIPPOCAMPAL REGION OF RATS AND MICE AFTER A SINGLE I.P. DOSE OF CLIOQUINOL: LOSS OF SYNAPTIC ZINC, CELL DEATH AND c-Fos INDUCTION

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Abstract—Clioquinol (CQ) is able to chelate synaptic zinc, which can modulate excitatory and inhibitory neurotransmission. In humans, CQ was associated with cases of transient global amnesia (TGA) and with the neurodegenerative syndrome subacute myelo-optico-neuropathy (SMON). We examined the CQ induced loss of synaptic zinc, cell death and c-Fos induction in rats and mice. In rats, we found a strong reduction of histochemically reactive synaptic zinc no later than 4 h after the injection of the lowest dose of CQ (50 mg/kg) and, for all doses used, a return to control levels after 48 h. There was no evidence of cell death for any dose and up to 1 week after CQ injections. Only a slight induction of c-Fos was seen in the hippocampus for the higher doses used (100–200 mg/kg). In mice injected with 100 mg/kg, CQ also resulted in a fast loss of synaptic zinc. c-Fos was induced after 4 h in cell populations of the hippocampal region and other parts of the telencephalon, and substantially increased after 24 h. One day after the injection we found a pattern of cell loss (hilus, parts of CA3, CA1 and layer III of the medial entorhinal cortex) reminiscent of that seen in models of temporal lobe epilepsy. In conjunction with published data on the behavioral effects of zinc chelation and the modulatory effects of zinc in excitatory neurotransmission, our results indicate that the loss of synaptic zinc may have been involved in TGA and the neuropathology associated with SMON.

Key words: hippocampus, c-Fos, excitotoxicity, SMON, subacute myelo-optico-neuropathy, transient global amnesia, temporal lobe epilepsy.

Clioquinol (5-chloro-7-iodo-8-hydroxyquinoline; CQ) was a component in medicines to treat for example traveler's diarrhea and other bowel symptoms. CQ acts as a chelator, which forms lipophilic chelates with cations like zinc, iron or nickel. In the 1960s, CQ was linked to subacute myelo-optic-neuropathy (SMON) in Japan (Kono, 1971). SMON symptoms included sensory disturbances, motor paralysis and loss of visual acuity, which correlated with axonal damage and demyelination of the optic nerve, lateral and posterior columns of the spinal cord and peripheral nerves. With the prohibition of CQ-containing medicines in 1970, the number of SMON cases in Japan decreased dramatically (Kono, 1975; Egashira and Matsuyama, 1982).

In addition to SMON, cases of acute encephalopathy similar to classic transient global amnesia (TGA) have been described after the intake of large doses of CQ. The CQ-type of TGA is characterized by an insidious start in cases mostly younger than those affected by the classic type. Patients were not able to retain what was shown or said to them, while they were otherwise able to act apparently normally and even to execute complex activities. During the acute amnesic episode, which could last up to 3 days (longer than in the classic type), there was also a retrograde amnesia, which could extend over months or years. After the episode of TGA, the capability to retain impressions returned and the retrograde amnesic gap was filled again. In both the classic and the CQ-type only the acute episode remained 'blank' (Ferrier and Eadie, 1973; Mumenthaler et al., 1979; Kaeser, 1984).

Although CQ was clearly the cause of the reported cases of TGA and almost conclusively established as the cause of SMON, a clear mechanistic link between cause and effect was never established. Waning interest in such a link after the virtual disappearance of SMON may in part be attributed to the fact that zinc only established itself in the arena of endogenous neuromodulators in the 1980s. Zinc is a potent modulator of excitatory neurotransmission effecting both NMDA and non-NMDA glutamate receptors (Harrison and Gibbons, 1994; Smart et al., 1994) through interactions with subunit specific zinc-binding sites (Kawajiri and Dingledine, 1993; Zheng et al., 1994; Paoletti et al., 2000; Choi et al., 2001; Rachline et al., 2005). While telencephalic GABAergic cells do not seem to use zinc as a neuromodulator (Pérez-Clausell and Danscher, 1985; Slomianka et al., 1997), effects of zinc on GABAergic transmission are robust and specific *in vitro* (Smart et al., 1994; Wang et al., 1995; Wooltorton et al., 1997; Hosie et al., 2003). Interactions of zinc released from excitatory boutons with GABAergic transmission or a colocalization of zinc and GABA outside the telencephalon seem possible (Birinyi et al., 2001; Wang et al., 2001). In addition to glutamatergic and GABAergic transmission, zinc has been found to modulate the function of receptors of most other major neurotransmitters *in vitro*. Recent evidence for a colocalization of zinc and glycine in the spinal cord (Birinyi et al., 2001) also support an *in vivo* role (Hirzel et al., 2006) of the modulation of glycinergic transmission by zinc (Laube et al., 1995; Miller et al., 2005).

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Abbreviations: Aβ, amyloid β; c-Fos+, c-Fos positive; CQ, clioquinol; NGS, normal goat serum; SMON, subacute myelo-optio-neuropathy; TBS, Tris-buffered saline; TGA, transient global amnesia; Zn-T3, zinc-transporter-3.

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Initially we became interested in the administration of CQ in animals that could serve as negative controls in histochemical studies of zinc-containing pathways (Slomianka et al., 1990) as large single doses (up to 400 mg/kg) of CQ did not show overt toxicity in rats (Kotaki et al., 1983). However, our first observations in mice and early literature on experimental findings in mice, dogs and cats, describing overt tonic–clonic seizures or seizure-like behaviors and associated histopathology in the hippocampus (Püschner and Fankhauser, 1969; Tateishi et al., 1973; Lannek and Jonsson, 1974; Krinke et al., 1978), prompted a more thorough reassessment of the effects of CQ in mice and rats. Effects of CQ may not only help to refine ideas on the function of zinc in the normal CNS, but are also of clinical interest. First, there are two case reports of acute seizures after CQ administration (Ogawa et al., 1975 as cited by Mumenthaler et al., 1979; Fisher et al., 1993) and two CQ-induced TGA patients—the only ones for whom a follow up is found in the literature—presented with temporal lobe epilepsy of unknown origin several years after the TGA episode (Ferrier et al., 1987). Second, interest in CQ and CQ-like compounds has re-emerged as a possible treatment for Alzheimer's disease. Amyloid β protein (Aβ) has binding sites for zinc (Bush et al., 1993), and the addition of zinc promotes the formation of insoluble Aβ deposits (Mantyh et al., 1993; Bush et al., 1994; Essler et al., 1996). Administration of CQ seems to be able to solubilize Aβ deposits in transgenic models of Alzheimer's disease (Cherny et al., 2001), and clinical trials were initiated to test for possible beneficial effects of CQ in Alzheimer's disease patients (Regland et al., 2001; Bush, 2002; Ritchie et al., 2003).

Here we report (1) the effect of intraperitoneally administered CQ on the distribution of zinc in the CNS of rats and mice at doses spanning the range from those that are likely to result in CQ-induced TGA in humans to doses that exceed those necessary to induce massive cell loss and seizures in mice, (2) histopathological changes associated with i.p. CQ administration in mice and (3) i.p. CQ-induced expression of the immediate early gene protein c-Fos as a marker for CQ-induced changes of neuronal function.

EXPERIMENTAL PROCEDURES

Animals

We used Wistar-Kyoto and Sprague–Dawley male and female rats, weighing from 82 to 232 g, and male DBA mice, weighing from 27 to 31 g. The animals were fed *ad libitum* and maintained under standard laboratory conditions of 24 °C and 12-h light/dark cycle. All animal experiments reported here were approved by the Animal Ethics and Experimentation Committee (AEEC) of the University of Western Australia and performed in accordance with the National Health and Medical Research Council (NHMRC) guidelines as outlined in the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

CQ administration

CQ was stirred thoroughly into castor oil (50 mg CQ/ml) until the suspension had a homogeneous, 'lotion-like' appearance. Suspensions were kept refrigerated and not used for more than 2–3 days. Rats were injected intraperitoneally with 50, 100 or 200 mg/kg CQ. Control animals were either injected with castor oil only (n=11) or left without treatment (n=4). Fifteen mice were injected intraperitoneally with 100 mg/kg CQ. Eleven mice were used as controls (castor oil only, n=7; no treatment, n=4).

For animal numbers and survival times following CQ injections see Table 1.

Table 1. Animals used by CQ dose and survival time

CQ (mg/kg)	Survival time after i.p. CQ				
	3–5 h	9 h	16 h	48 h	168 h
Rats					
0	9+3 SD		1		2
50	4				
100	11+3 SD	3	1	3	5
200	5		1		2
Mice					
	3–4 h		24 h		
0	8		3		
100	5		10		

SD: Sprague–Dawley rats, otherwise Wistar-Kyoto rats or DBA mice. All animals survived the CQ-dose for the specified survival times.

Tissue processing

To demonstrate zinc using Timm's sulfide silver method (Danscher, 1981; Geneser et al., 1993), animals were deeply anesthetized with sodium pentobarbital and perfused transcardially with 0.5% sodium sulfide in 0.15 M phosphate buffer (SPB; pH 7.4) for 2 min at a pressure of ~120 mm Hg prior to 4% PFA in 0.15 M phosphate buffer (pH 7.4) at room temperature for 6–8 min. To demonstrate vesicular zinc using the selenium method, 20 mg/kg Na_2SeO_3 in H_2O (1 ml/100 g) was injected i.p. under a light anesthesia 1 h prior to pentobarbital anesthesia and perfusion with 4% PFA.

Brains were postfixed for 2 h, cryoprotected in 30% sucrose and frozen. Serial 40 μm sections were cut and either mounted on slides or collected into a cryoprotectant to be stored at −30 °C until further processing.

Slides were immersed in a developer (Danscher, 1981; Geneser et al., 1993) made from 60 ml of protective colloid (50% w/v gum arabic in deionized water) and 10 ml citrate buffer (25.5 g citric acid$\times H_2O$ and 23.5 g sodium citrate$\times 2H_2O$ dissolved in deionized water to a total volume of 100 ml). Freshly prepared solutions of 0.85 g hydroquinone in 15 ml deionized H_2O and 0.12 g silver lactate dissolved in 15 ml deionized H_2O were stirred into the developer immediately before use. Sections were developed at 26 °C in the dark for 60–70 min.

To demonstrate c-Fos, free-floating sections were rinsed, incubated for 1 h in 1% Triton in Tris-buffered saline (pH 7.4; TBS; 0.9% NaCl), rinsed and incubated for 1 h in TBS containing 4% normal goat serum (NGS) and 2.5 mg/ml bovine serum albumin. After additional rinses, sections were incubated for 24 h at room temperature in primary rabbit c-Fos antibody (PC38; Oncogene, Cambridge, MA, USA) at a dilution of 1/40,000 in TBS with 1% NGS. Thereafter, sections were rinsed and incubated in secondary biotinylated goat anti-rabbit antibodies (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA, USA) at 1/200 in TBS and 1% NGS for 1 h. Finally, sections were again rinsed, incubated in avidin–biotin complex for 20 min and stained using diaminobenzidine as chromogen. Omission of the primary antibody from the incubation solutions abolished immunoreactivity with the exception of a faint, homogeneous background stain.

One series from each animal was Nissl stained using acidic Toluidine Blue.