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Suppression of spike-wave discharge activity and *c-fos* expression by 2-methyl-4-oxo-3H-quinazoline-3-acetyl piperidine (Q5) *in vivo*

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Abstract

Antiepileptic and network inhibitory actions of Q5 (2-methyl-4-oxo-3H-quinazoline-3-acetyl piperidine) have recently been described in hippocampal slices. Here we present evidence on the *in vivo* antiabsence effect of Q5. All doses of Q5 tested (0.3 mg/kg, 0.9 mg/kg, 2.8 mg/kg) decreased the number, but not the duration and the frequency of absence spike-wave discharges (SWDs) in freely moving WAG/Rij rats. *In vivo* network inhibitory action of Q5 was monitored by following *c-fos* expression in different brain areas of Wistar rats. Significant depletion of *c-fos* expression was observed after single or repeated injections of Q5 (2.8 mg/kg and 2 × 2.8 mg/kg) in various brain areas, including hypothalamic paraventricular nucleus, medial amygdaloid nucleus, piriform cortex, somatosensory cortex, periventricular thalamic nucleus and periaqueductal central gray. Thus, our *in vivo* results demonstrate that in addition to the prevention of absence seizures, Q5 effectively suppresses neuronal activation in various stress- and pain-sensitive brain areas.
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Keywords: 2-Methyl-4-oxo-3H-quinazoline-3-acetyl piperidine; WAG/Rij rat; Spike-wave discharge; *c-fos* expression

In the present study *in vivo* effects of the potential anticonvulsant, 4-oxo-2-methyl-4-oxo-3H-quinazoline-3-acetyl piperidine (Q5) have been addressed. Previous reports based on *in vitro* measurements have shown that Q5 is endowed with anticonvulsant efficacy, mediated by the depression of network excitability *via* some metabotropic Glu receptor (mGluR) related mechanisms undefined as yet [12,13]. Furthermore, the water–lipid partition coefficient of the compound [1] suggests that this compound might easily cross the blood–brain barrier and thus might be effective *in vivo* as well. The Wistar Albino Glaxo/Rijswijk (WAG/Rij) rat strain is recognized as an animal model for human absence epilepsy generating a genetically determined form of SWDs [5]. In addition, extensive pharmacological studies substantiate the use of WAG/Rij model for testing potential antiepileptic drugs [5]. Although neurochemical and neuroanatomical origin of generalized convulsive and absence seizures are different, similar effects of mGluR ligands have been observed in convulsive and absence models [5,18]. Here we present the *in vivo* effects of intraperitoneal (i.p.) Q5 injection on SWDs in WAG/Rij rats. To characterize network inhibition *in vivo*, effects of Q5 on neuronal activation have been explored using the inducible immediate-early gene *c-fos* as a marker to reveal activation of neurons in various brain areas [9,11,25]. Here we report the effects of Q5 injection on *c-fos* expression in 15 different brain areas of Wistar rats.

Q5 (kindly obtained from József Kökösi, Institute of Pharmaceutical Chemistry, Semmelweis University, Hungary) was dissolved in artificial cerebrospinal fluid (ACSF: 129 mM NaCl, 3 mM KCl, 1.6 mM $CaCl_2$, 1.8 mM $MgSO_4$, 21 mM $NaHCO_3$, 1.23 mM NaH_2PO_4, pH 7.4). Unless otherwise stated below, all

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chemicals were purchased from Merck (Budapest, Hungary). The animals were kept in standard laboratory conditions and the experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC), Animal Act 1998, Hungary and local ethical rules.

Adult WAG/Rij (7–9 months old; $n = 15$; weight: 280–330 g) rats were implanted in Halothane-air mixture (1%) anesthesia with 0.8 mm stainless screw electrodes for EEG recording. Screws were placed into the skull above the frontal (A: 2.0; L: 2.1) and parietal (A -6.5; L 2.1) cortex according to the atlas of Paxinos and Watson [26]. The ground electrode was placed above the cerebellar cortex. Electrodes were soldered to a ten-pin socket and glued to the skull with dentacrylate cement. Rats were allowed to recover for at least 2 weeks controlled by recording the EEG. Animals were gently handled several times daily to reduce stress-induced changes of SWD number. EEG were recorded in the animal home cage by a differential preamplifier (SUPERTECH Bioamp 4, Hungary) attached to a CED 1401 mkII a data capture and analysis device using SPIKE 2 software of the company (Cambridge, UK). The bandwidth of the EEG recording was 0.53–75 Hz and it was sampled at a 500 Hz sampling rate. SWDs were marked at visual inspection based on commonly used criteria: a typical SWD was defined as a train of asymmetric spikes and slow waves starting and ending with sharp spikes with a dominant frequency of 7–11 Hz. SWD amplitude was at least twice as high as the EEG delta wave activity, the duration of SWDs was longer than 1 s [32]. Selected SWDs were checked by Fourier and wavelet transform analysis [24].

Rats were divided into three groups ($n = 5$, for each dose) and received 0.3 mg/kg, 0.9 mg/kg and 2.8 mg/kg Q5 i.p. The cortical EEG was recorded between ipsilateral fronto-parietal electrodes in freely moving animals from 4 p.m. to 7 p.m. because of the circadian rhythm of SWD genesis [7]. In each animal, control injections were applied on three consecutive days, in the same period of time, before the application of Q5 on the fourth day. Alterations in SWD number and duration were measured during the 180 min post-injection period. Data were pooled from every 30 min periods. SWD data of control injections have been averaged (control). SWD data of Q5 injection are given as percentage of control. Statistical significance of results was obtained using one-way analysis of variance (ANOVA).

Effects of i.p. application of Q5 were investigated on *c-fos* expression in various forebrain and midbrain areas of male Wistar rats (165–285 g body weights; $n = 20$). Q5 (0.5 mM, 1.425 mg/10 ml) was dissolved in 0.9% NaCl. The animals were divided into four experimental groups ($n = 5$ in each group), and injected i.p. as follows: (1) control (1 × NaCl): 2 ml 0.9% NaCl/100 g body weight; (2) 2 ml 0.5 mM Q5/100 g body weight (1 × 2.8 mg/kg); (3) control (2 × NaCl): 2 ml 0.9% NaCl/100 g body weight, and it was repeated 1 h later. (4) 2 × Q5: 2 ml 0.5 mM Q5/100 g body weight twice as control rats. Animals of group No. 1 and No. 2 were perfused with 4% paraformaldehyde one hour after the first, while rats of group No. 3 and No. 4 1 h after the second injection. Brains were removed, postfixed in the same fixative solution, were cut in the coronal plane (60 μm thick serial sections). Immunohistostaining was performed on free-floating sections using the avidin–biotin peroxidase (ABC, Vectastain) method. Briefly, sections were washed in 0.1 M phosphate-buffered saline (PBS), incubated in Triton X-100 and in 10% normal goat serum, and subsequently incubated for 48 h with specific *c-fos* antibody (Santa

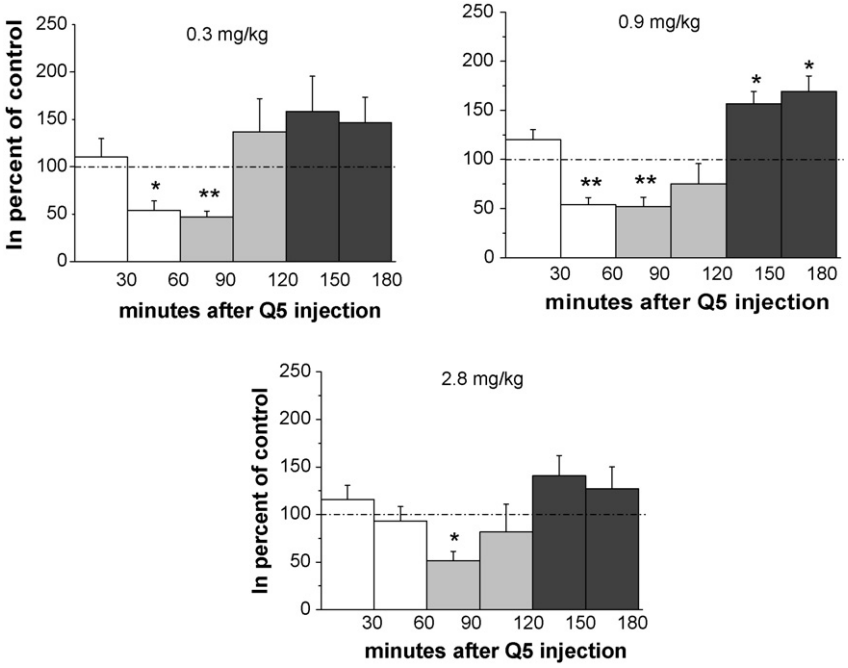

Fig. 1. Changes in SWD numbers after intraperitoneal injection of different doses of Q5 (0.3 mg/kg, 0.9 mg/kg, 2.8 mg/kg). Number of SWDs were pooled in every 30 min and expressed as the percentage of control values calculated as the average SWD number of the same time period measured after ACSF injection. $^{*}p < 0.05$, $^{**}p < 0.005$ level of significance.

Cruz, 1:10,000). The next step was incubation in secondary anti-rabbit antibody for 1 h and in avidin–biotin peroxidase for 1 h. Phosphate-buffered saline (0.1 M, pH 7.26) was used to wash sections between all steps. The antigen-antibody complexes were visualized by 3,3′-diaminobenzidine and 0.03% H_2O_2. Light counter-staining with Kernechtrot dye (Chroma, Köngen/N, Germany) was applied. Sections with 13 forebrain and 2 midbrain regions were used for quantitative analysis of *c-fos* activity, as reported previously [25]. The average density of Fos-positive cells in selected areas was determined by counting the Fos-positive cells using a rectangular grid (0.01 mm^2) placed randomly on the investigated areas (4–6 times/section) under a projection microscope. Data from four sections of each investigated areas from both sides ($n = 4 \times 4 \times 2 = 32$) were averaged for each animal (mean ± S.E.M.).

Representative SWDs showed the dominant power peak at 8 Hz and some of upper harmonics (data not shown). Results of wavelet analysis indicated that application of any dose of Q5 did not change the frequency components and dynamics of SWD activity (data not shown). Both the number and the duration of SWD episodes obtained in control experiments varied in a wide range individually (number: 5–27 within 30 min, duration: 3.4–29 s). Thus, data have been normalized to the average of data obtained on control (ACSF injected) days. Application of the lowest and the medium doses of Q5 (0.3 mg/kg and 0.9 mg/kg) resulted in a transient significant decrease (about 50% of control) in the number of SWD episodes at 30–90 min after injection (Fig. 1). The SWD number was significantly decreased 60–90 min after a 2.8 mg/kg dose of Q5 injection. In contrast, significant increase in SWD number was found 150–180 min after application of 0.9 mg/kg Q5 (Fig. 1). Durations of individual SWD episodes did not change significantly during the application of any dose of Q5 throughout the experiment (data from the most effective dose (0.9 mg/kg): 96 ± 4.6%, 98 ± 11%, 89 ± 7.7%, 93 ± 6.7%, 99 ± 6.5%, 96 ± 6.3%). Injection of 2 ml Q5 in ACSF caused a short increase of behavioral excitation (30–50 s) characterized by air sniffing, rearing and grooming, however, these were not distinguishable from that evoked by ACSF. Other changes in the behavior or movement were not observed during the recorded periods.

One hour after i.p. injection of saline, very high density of Fos-positive cells were observed in the somatosensory cortex, relatively high density in the supramamillary and paraventricular nuclei of the hypothalamus, the medial amygdaloid nucleus, the midbrain central gray matter and the piriform cortex (Fig. 2). In addition, moderate to high density of Fos-positive neurons were counted in the midline (periventricular) thalamic nuclei, the subparafascicular nucleus, the anterior and dorsomedial nuclei in the hypothalamus and the arcuate nucleus. In contrast, cells in the central nucleus of the amygdala, dentate gyrus and the hippocampus did not establish any marked Fos reaction in response to either single or repeated i.p. injection of saline (Fig. 2).

The density of Fos-positive neurons was significantly lower after a single Q5 injection than that after the saline application in brain areas including the hypothalamic paraventricular nucleus, somatosensory cortex, central amygdaloid nucleus and

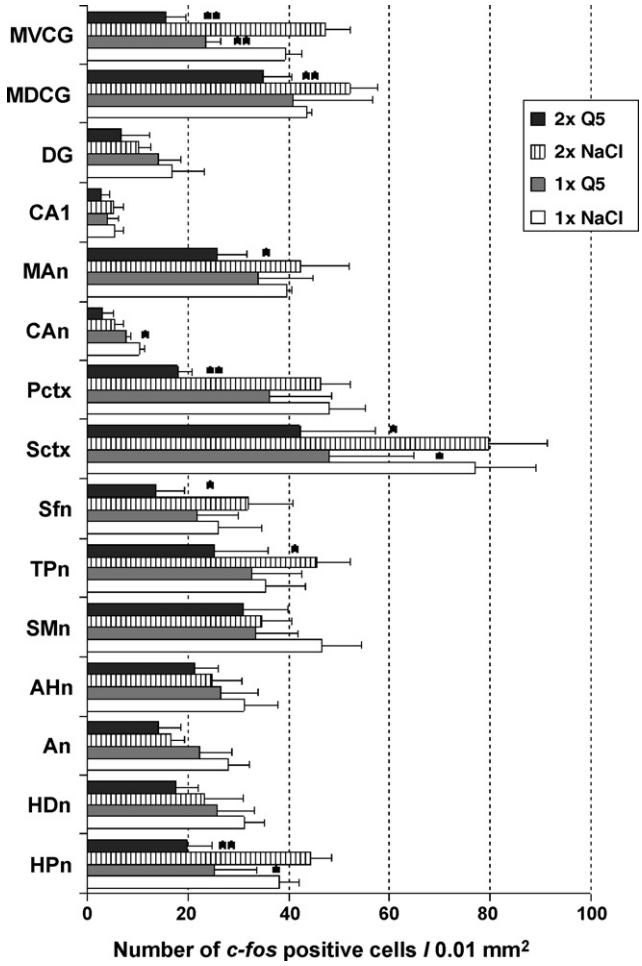

Fig. 2. Effects of single (dark grey bars) and double (black bars) intraperitoneal injections of Q5 (2.8 mg/kg and 2 × 2.8 mg/kg i.p.) vs. single (white bars) and double (striped bars) injections of saline on the number of *c-fos* positive cells in 15 brain areas (0.01 mm^2) measured. MVCG—midbrain ventral central gray, MDCG—midbrain dorsal central gray, DG—dentate gyrus (stratum granulosum), CA1—hippocampus CA1 (pyramidal layer), MAn—medial amygdaloid nucleus, CAn—central amygdaloid nucleus, Pctx—piriform cortex, Sctx—somatosensory cortex, Sfn—Subparafascicular nucleus, TPn—thalamic periventricular nucleus, SMn—supramamillary nucleus, AHn—anterior hypothalamic nucleus, An—arcuate nucleus, HDn—hypothalamic dorsomedial nucleus, HPn—hypothalamic paraventricular nucleus. Significance marks indicate that Q5 group was compared to saline injected group (*t*-test) $^{*}p < 0.05$, $^{**}p < 0.005$.

midbrain central gray matter (Fig. 2). The density of Fos-positive neurons was even lower after repeated injections of Q5 that reached the significance level in several brain areas: hypothalamic paraventricular nucleus, somatosensory cortex, thalamic periventricular nucleus, subparafascicular nucleus, piriform cortex, medial amygdaloid nucleus, and both in the dorsal and ventral parts of the periaqueductal central gray (Fig. 2). These changes were particularly high in the paraventricular nucleus, the somatosensory and piriform cortex and in cells of the ventral part of the periaqueductal gray. Neither single nor repeated injection of Q5 altered Fos-positive cell density compared to saline injected control rats, in the hippocampus (dentate gyrus and CA1 pyramidal layer) and the hypothalamic dorsomedial, anterior, supramamillary and arcuate nuclei (Fig. 2).

Absence seizures are generated via the cortico-thalamo-cortical neuronal circuit with participation of the reticular, ventral posteromedial thalamic nucleus and the cerebral cortex [5,28]. Sustained synchronous activity of thalamic relay cells and T-type calcium channel dependent calcium spikes have a crucial role in SWD genesis, similarly to the mechanisms of alpha waves and sleep spindles [5,21,28,29]. It has been reported that the direction of cortico-thalamic interactions could change throughout one seizure: the peri-oral region of the somatosensory cortex drives the thalamus during the first 500 ms of the seizure and subsequently the thalamocortical oscillatory network maintains SWDs [5,21]. Recent studies supported the key role of the somatosensory cortex in initiating absence seizures since application of ethosuximide or lidocaine into the peri-oral region of the somatosensory cortex significantly decreased the number of SWDs in genetic absence epilepsy rats from Strasbourg (GAERS) and in WAG/Rij rats, respectively [17,27]. We found, that all of the doses of Q5 applied suppressed the number (Fig. 1), but not the duration of individual SWDs indicating that Q5 has an inhibitory effect on the SWD-initiation process. Accordingly, *c-fos* expression observed in the somatosensory cortex after Q5 application suggests an inhibitory action of Q5 on these cortical neurons *in vivo*. It is not clear yet, how Q5 could generate a delayed facilitation of SWD number between 120–180 min after injection. Modulation of other neurotransmitters involved in SWD genesis by Q5 *via* mGluRs offer a possible explanation for these results [2,15,19,28]. Alternatively a mixed effect of Q5 on mGluRs might explain these observations.

After i.p. injection of saline (as a moderate, short lasting stressful stimulus), a number of Fos-immunopositive cells appeared in stress-sensitive hypothalamic and limbic nuclei, as well as in limbic and somatosensory cortical areas and in different cell groups in the midbrain central gray (Fig. 2). In addition, moderate to high density of Fos-positive neurons were counted in pain-related brain areas like in the somatosensory cortex, the midline (periventricular) thalamic nucleus and the periaqueductal central gray. In general, application of Q5 significantly decreased *c-fos* expression. Since increased *c-fos* expression may reflect neuronal activation accompanied by increased intracellular calcium ion concentration [11], the inhibitory effect of Q5 on *c-fos* expression *in vivo* are in line with decreased intracellular calcium transients and network inhibition measured after application of Q5 *in vitro* [13]. Among the brain areas where Q5 decreased *c-fos* expression the somatosensory cortex had been reported to be involved in the control of SWDs. Due to the absence of *c-fos* induction in the reticular and ventral posteromedial thalamic nucleus by i.p. injection we may not include the involvement of these areas in the antiabsence effect of Q5. Furthermore, based on various studies confirming the brain structures involved in the generation or modulation of SWDs [5,17,21,27,31], the significant contribution of other brain areas where Q5 effectively decreased *c-fos* expression does not seem likely.

Worthy of attention is the decreased number of *c-fos* positive cells in specific stress- and pain-related areas by Q5 application raising the question of possible anxiolytic and/or analgesic effects of Q5. Collectively, our data suggest that Q5 is an effective antiabsence compound, and it suppresses neuronal activation in specific brain areas *in vivo*.

Although the target of Q5 action has not been identified yet, recent *in vitro* results suggested that Q5 may possibly act through some mGluR subtype [13]. It is to note in this respect, that the involvement of mGluRs in thalamocortical oscillations [10,20] and in neuronal responses to stress, anxiety and pain [14,30,33] has been observed previously. Specific antagonists of either group I mGluRs [3,4] or group II/III mGluRs [6,22,23] have been found to reduce absence epileptic activity *in vivo* (doses: 1–50 mg/kg). In models of pain, anxiety and stress [14,30,33] antagonists of group I mGluRs but also agonist of group II/III mGluRs were reported to be effective. Furthermore, inhibitory effects of group I antagonist on *c-fos* expression have been reported in some brain areas, including the striatum [8,34] and the hippocampus [16]. Conversely, a group II mGluR antagonist, as the antiabsence LY341495 [23] has been demonstrated to induce *c-fos* expression in stress-related brain regions [14]. Comparing the related *in vivo* effects of Q5 and specific mGluR ligands may support the view suggested by *in vitro* studies [12] that Q5 acts through some mGluRs. In conclusion, we found Q5 to be effective in suppression of absence epileptic activity as well as decreasing moderate stress- and pain-induced *c-fos* expression *in vivo*.

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