

Importance of NO/cGMP signalling via cGMP-dependent protein kinase II for controlling emotionality and neurobehavioural effects of alcohol

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Abstract

Cyclic GMP is a second messenger for nitric oxide (NO) that acts as a mediator for many different physiological functions. The cGMP-dependent protein kinases (cGKs) mediate cellular signalling induced by NO and cGMP. Here, we explored the localization of cGMP-dependent protein kinase type II (cGKII) in the mouse brain. *In situ* hybridization revealed high levels of cGKII mRNA in cerebral cortex, thalamic nuclei, hypothalamic nuclei, and in several basal forebrain regions including medial septum, striatum and amygdala. The close link to NO and the distribution pattern of cGKII suggested that this enzyme might be involved in emotional reactions and responses to drugs of abuse. Therefore, cGKII knockout animals (cGKII^{-/-}) were compared with littermate controls in behavioural tests (i) for emotion-linked and (ii) for acute and chronic ethanol responses. Deletion of cGKII did not influence aggressive behaviour but led to enhanced anxiety-like behaviour. In terms of acute responses to ethanol, cGKII^{-/-} mice were hyposensitive to hypnotic doses of ethanol as measured by the loss of righting reflex, without an alteration in their blood alcohol elimination. In a two-bottle free choice test, cGKII^{-/-} mice showed elevated alcohol consumption. No taste differences to sweet solutions were observed compared to control animals. In summary, our data show that cGKII activity modulates anxiety-like behaviour and neurobehavioural effects of alcohol.

Introduction

Nitric oxide (NO) is an intra- and extracellular messenger, which is produced by nitric oxide synthase (NOS), and which affects various behaviours in rodents (Hofmann *et al.*, 2003). Male mice treated with inhibitors of neuronal NO synthase (nNOS) displayed a marked increase in aggressive and sexual behaviour (Demas *et al.*, 1997) and a decrease in anxiety-like behaviour (Yildiz *et al.*, 2000; Volke *et al.*, 2003). Similar effects are also observed following targeted disruption of the gene for nNOS (Nelson *et al.*, 1995; Chiavegatto *et al.*, 2001; Bilbo *et al.*, 2003; Weitzdoerfer *et al.*, 2004).

The relationship between alcohol consumption and aggressive behaviour (Jaffe *et al.*, 1988; Moeller *et al.*, 1998; Pihl & LeMarquand, 1998; Fish *et al.*, 2002), as well as anxiety-like behaviour (Spanagel & Hölter, 1999; Henniger *et al.*, 2002), suggests that NO signalling might be involved in the alcohol drinking behaviour. In fact pharmacological intervention studies using unselective NOS inhibitors showed an attenuation of alcohol consumption in alcohol-preferring rats (Rezvani

et al., 1995), in Sprague–Dawley rats selected for high alcohol intake (Calapai *et al.*, 1996), and in rats that underwent chronic alcohol intoxication prior self-administration experiments (Lallemand & De Witte, 1997). In contrast, activation of the NO pathway opposes the effects of acute ethanol administration in rats (Ferreira *et al.*, 1999) raising the possibility that NO could act as a feedback inhibitory loop following exposure to ethanol. In line with this hypothesis, it was recently found that genetic deletion of nNOS increased alcohol consumption in mice (Spanagel *et al.*, 2002).

The activation of the guanylyl cyclase and the resulting elevation of cyclic cGMP is a major downstream signal of NO in neurons. The signalling pathway after cGMP synthesis is not obvious, as cGMP affects several ion channels and phosphodiesterases *in vivo*. In many cells, the target of cGMP is the cGMP-dependent protein kinase I or II, abbreviated as cGKI or cGKII, respectively (Hofmann *et al.*, 2000). cGKI is highly expressed in cerebellar Purkinje cells, hippocampal neurons and bulbus olfactory neurons whereas cGKII has a much wider distribution in the rat brain (El-Husseini *et al.*, 1995; De Vente *et al.*, 2001). In the brain, NO, cGMP and cGKII are close related because both enzymes, nNOS and cGKII, frequently are coexpressed either directly, or indirectly with cGKII-expressing neurons, which receive afferents from nNOS-containing neurons (El-Husseini *et al.*, 1999).

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In the current study, we first explored the expression pattern of cGKII in mouse brain. The detection of cGKII mRNA in the mouse cerebral cortex, thalamic nuclei, hypothalamic nuclei, and in several basal forebrain regions and the tight link to NO led us to predict that cGKII might be involved in emotional reactions such as aggression and anxiety behaviour. Therefore, we examined the effects of germ line disruption of the cGKII gene on emotional behaviour with special emphasis on aggressive behaviour and anxiety-like behaviour. As these behaviours and NO are also linked to acute and chronic responses to alcohol we further studied neurobehavioural effects of alcohol in cGKII-deficient mice.

Materials and methods

Animal breeding

The generation of cGKII-knockout (cGKII^{-/-}) has been described previously (Pfeifer *et al.*, 1996). cGKII^{-/-} mice were either kept on the Sv/129 background or back-crossed to C57BL/6N for nine generations. All experiments were performed in cGKII^{-/-} and cGKII^{+/+} mice on a pure C57BL/6N background. However, cGKII^{-/-} and cGKII^{+/+} mice on Sv/129 background were also used for measuring acute and chronic effects of ethanol, as previous studies showed that genetic background can have a profound effect on ethanol-induced loss of righting reflex (LORR; Cowen *et al.*, 2003) and alcohol drinking measurements (Spanagel *et al.*, 2002; Wahlsten *et al.*, 2003). The animals were kept under standard housing conditions (21 ± 1 °C, 55 ± 10% relative humidity, 12-h dark : 12-h light cycle). All investigations were performed on adult male mice (> 8 weeks old) that were either group-housed or for measurements of aggressive behaviour, voluntary ethanol consumption, and taste preference tests, single-housed in standard (type 2) hanging rodent cages with food and water *ad libitum*. If not otherwise indicated, in most experiments 14–20 mice per group were studied per strain (Sv/129 or C57BL/6 N). The experiments were performed in different animal groups with one exception; the groups of animals that underwent the voluntary ethanol consumption procedure were used later on for the measurement of the hypnotic effects of ethanol. The animal experiments were conducted in accordance with the European Communities Council Directive of November 24th 1986 (86/EEC) and were approved by the Governments of Upper Bavaria, Germany and Baden-Württemberg.

Drugs

Ethanol-drinking solutions were made up from 96% ethanol diluted with tap water to the different concentrations. For injections, 96% ethanol was diluted with 0.9% saline to a 12.5% (v/v) solution. Sucrose and saccharin were obtained from Sigma (Deisenhofen, Germany) and were diluted with tap water.

mRNA in situ hybridization

Adult mice of the strain C57BL/6N were anaesthetized with sodium pentobarbital and perfused with 50 mL ice-cold PBS, pH 7.4. The brain was removed and quickly frozen in isopentane cooled in a dry ice/ethanol bath. Sections (20-µm thick) were cut in a cryostat (Leica, Jung CM 3000), thaw-mounted onto Super FrostTM Plus-slides (Menzel-Gläser, Germany), fixed with 4% paraformaldehyde in PBS, pH 7.4, and acetylated with 0.25% acetic anhydride (Merck, Darmstadt, Germany) in 0.1 M triethanolamine, pH 8.0, for 10 min. The slides were dehydrated in several changes of ethanol, isopropanol

and xylene, dried and then prehybridized for 2 h at 42 °C in hybridization buffer (10 mM Tris, pH 8.0, 0.3 M NaCl, 1 mM EDTA, 1 × Denhardt's solution, 10% dextran, 50% deionized formamide, 50 mM DTT). For *in situ* hybridization, a cDNA of cGKII from mouse brain (Uhler, 1993; nucleotides 960–1740) was cloned into the PAL1-Vector (Ludwig *et al.*, 1997), radiolabelled with [³⁵S]-UTP (NEN, Amersham) using the reverse transcription kit (Stratagene Europe, Amsterdam) and then incubated with prehybridized sections at a concentration of 5 × 10⁶ cpm/mL hybridization buffer for 16 h at 55 °C. After hybridization, the slides were washed two times in 2 × SSC, 1 mM DTT, 1 mM EDTA, incubated in RNase A (20 µg/mL, Life Technologies, Karlsruhe) for 30 min at room temperature and then washed twice in 2 × SSC, 1 mM DTT, 1 mM EDTA. The sections were then washed at high stringency in 0.1 × SSC, 1 mM DTT, 1 mM EDTA for 2 h at 75 °C, dehydrated, dried, and exposed to BioMax MR (Kodak, Rochester, NY) film for 6 days. The slides were next dipped in autoradiography emulsion NTB-2 (Kodak), exposed for 6–8 weeks, and developed in D-19 developer (Kodak). Sections were lightly counterstained with haematoxyline/eosin and examined with bright- and dark-field illumination. Photomicrographs were taken at 20× or 40× magnification using a Kodak Select 400ASA-film. Cerebral structures and nuclei were identified according to Paxinos & Watson (1986).

Basal locomotion in an open field

Mouse locomotion was tested in a modified open field box of 30 × 30 × 40 cm. A video motility system (TSE-System, Bad Homburg, F.R.G.) provided the activity measurement of six animals simultaneously and basal activity (cm/min) was detected for 30 min in 5-min intervals.

Measurement of aggressive behaviour

cGKII^{-/-} and control mice were studied by a resident/intruder interaction test as described by Krsiak (1976) and Miczek *et al.* (1998). Social interactions always involved one single-housed (cGKII^{-/-} or wild-type littermate) and one weight-matched group-housed mouse, being placed as pairs in the observational cages (20 × 30 × 20 cm). After 3 weeks of isolation the social interaction was performed. The isolates were allowed 30-min adaptation to the observational cages before the group-housed partners were introduced; the interaction ended after 4 min. The observational cages were cleaned and their floors were covered with new wood shavings after each interaction. The behaviour of animals during the interaction was recorded on videotape. Tapes were then analysed by an observer blind to treatment and genotype. This was performed with a keyboard that was connected to a standard PC and software for behavioural analysis (Observer, Noldus, Freiburg, Germany). The frequency, total duration and latency of a number of aggressive activities were recorded. In short, the acts and postures evaluated were defined as follows: attack, a fierce lunging at the partner often associated with biting; and tail rattle, rapid vibrations of the tail. Complete lack of attacks towards the partner, accompanied by specific 'submissive' postures, escape and defense was regarded as submissive type of social behaviour.

Analysis of anxiety-like behaviour

Anxiety-like behaviour was analysed in the light-dark box test as described previously (Timpl *et al.*, 1998). The light-dark box is a plexiglass maze with two compartments, a 15 × 20 × 25 cm dark

(sheltered) compartment, and a 30 × 20 × 25 cm lit compartment, the latter brightly illuminated (600 lux) and therefore aversive. Both compartments are connected by a 4-cm long dark tunnel. During the test the animals' behaviour was recorded for 5 min using a video camera system. The number of visits and the time spent in the aversive, i.e. the lit compartment was analysed and taken as a correlate of anxious behaviour.

The elevated O-maze consisted of a black circular path (runway width 5.5 cm, diameter 46 cm) that was covered with fleece paper and placed 20 cm above the floor, illumination intensity was 5 lux. Two opposing compartments were protected by walls of grey polyvinylchloride (height 10 cm). Mice were introduced to one of the two closed compartments. The number of exits to the anxiety-like open compartments of the maze and total duration of time spent therein were scored during a 5-min observation period.

Measurement of the loss of righting reflex (LORR)

A single dose of 4.0 g/kg ethanol was injected intraperitoneally (i.p.) in animals derived from the 129/SvN and the C57BL/6N strain. When animals became ataxic, they were placed on their back in a V-shaped paper trough and the time was recorded until the righting response. Animals were judged to have regained their righting response when they could right themselves three times within 30 s. The observer was blind to the experimental design. The time between the injection and the onset of LORR was measured, as well as the recovery time.

Measurement of ethanol clearance

The mice were injected i.p. with 3 g/kg body weight of ethanol and small blood samples were withdrawn 0.5, 1.5, 2.5, and 3.5 h after injection by retroorbital venous plexus puncture. Blood ethanol concentration was determined photometrically, at 340 nm, by the turnover of NAD to NADH in the presence of alcohol dehydrogenase (Kit 332 UV, Sigma, Deisenhofen, Germany).

Voluntary ethanol consumption

For 3 weeks cGKII-deficient mice and wild-type littermates had a free choice between a bottle with tap-water or a bottle with a 5% ethanol solution (w/v). Bottles were weighed daily at 10:00 h and all drinking solutions were renewed every 3 days. The positions of the two bottles were changed every 3 days to avoid side preferences.

Taste preference tests

Alcohol-naïve and alcohol-experienced mice were used for these tests. A pilot study showed that alcohol self-administration for two months had no influence on subsequent intake of sweet tasting solutions when compared to age-matched alcohol-naïve mice (see also Spanagel *et al.*, 2002). Sucrose (4%) and saccharin (0.06%) solution intake was measured in a two-bottles free choice test (against tap water). A test lasted for 6 days and bottles were weighed every 3 days and the position of the bottles was then changed.

Statistics

All data are presented as the mean ± the standard error of the mean (SEM) and a significance level of $P < 0.05$ was used throughout. In

general, direct comparisons were made either by simple *t*-tests or by analysis of variance (ANOVA) and where appropriate Newmann-Keuls' *posthoc* test was applied.

Results

cGKII in the mouse brain is expressed in regions involved in emotionality and drug responsiveness

Initial immunoblot analysis of mouse brain extracts indicated that cGKII protein was present in almost all brain areas. The available antibodies did not work satisfactorily in immunohistochemical tests using brain slices from wild-type and knockout animals. We therefore explored the neuronal expression of cGKII using *in situ* hybridization (see Figs 1 and 2 and Table 1 for a summary). High levels of cGKII mRNA were observed in parts of the cortex, olfactory bulb, thalamus and basal forebrain, as well as the midbrain raphe nuclei and brainstem sensory regions. In the olfactory bulb, very strong signal was observed over the periglomerular neurons, more moderately on the mitral cells and intermediate in the external part of the granular layer (Fig. 1A). In the cortex, strong labelling was restricted to upper layers, particularly the most superficial part of layer 2 (Figs 1C–J, and 2D, F and G). Cortical regions were not labelled uniformly, with very high levels in the piriform cortex (Fig. 2H), high in the entorhinal cortex (Fig. 2G), high to intermediate in the somatosensory and motor cortex and visual cortex (Figs 1D–J, and 2F), moderate in frontal and anterior cingulate (Figs 1A–C, and 2D), and low in the subiculum and the retrosplenial agranular cortex (Fig. 1H–J). In the extensively stained somatosensory and visual cortex, the strong cGKII mRNA expression extended down into layer 4 (Figs 1D–J, and 2F), in the more moderately labelled frontal and anterior cingulate it was largely confined to the superficial part of layer 2 (Fig. 2D). The hippocampal expression of cGKII was very low (Figs 1E–I, and 2I).

As shown in Figs 1G–I, and 2H, the amygdala displayed moderately strong but uneven labelling. Uneven distribution of cGKII mRNA was also observed in the thalamus (Fig. 1E–H), with strong labelling of the anterior dorsal nuclei, moderate in the medial and lateral geniculate, weak in the habenulae, the ventrolateral group, other thalamic nuclei and nucleus subthalamicus. In other parts of the forebrain, high levels of cGKII mRNA were also observed in the septohippocampal nucleus (Fig. 2B), the diagonal band of Broca (Fig. 2C) and in the preoptic and periventricular hypothalamic nuclei (Figs 1D–F, and 2E). In the mid- and hind-brain, strong cGKII mRNA expression was present in the midbrain dorsal raphe nuclei and the mesencephalic trigeminal neurons (Fig. 2J), the solitary tract nucleus (Fig. 2K) and the spinal nucleus of the trigeminal nerve (not shown). Most parts of the brain stem, including cerebellum, area postrema and motor nuclei showed little cGKII mRNA expression (Figs 1K, and 2K).

cGKII knockouts do not differ in aggressive behaviour but show higher anxiety-like behaviour than wild-type littermates

In the resident/intruder interaction test measures of aggression (attacks and tail rattles) were taken in terms of frequency and duration of these behaviours and latency to perform these behaviours (Fig. 3). While there was some variation between the animals, there were no significant differences in terms of frequency, duration or latency of attacks or tail rattles when cGKII knockouts were compared with wild-type littermates (Fig. 3). In terms of submissive behaviour no significant differences between genotypes could be observed (data not shown).

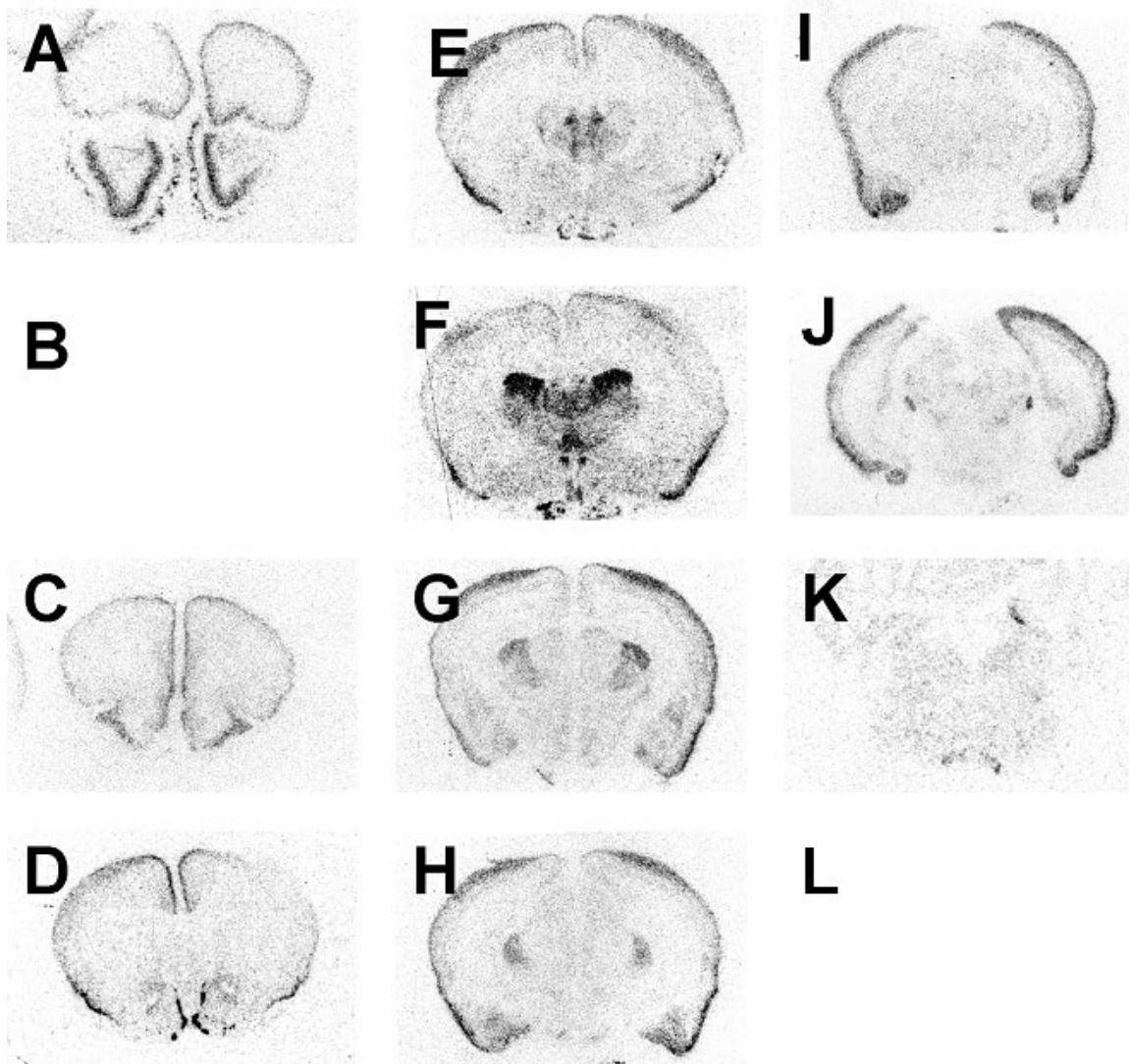


FIG. 1. cGKII mRNA expression in mouse brain. Film autoradiography in sequential mouse brain sections in rostrocaudal direction from olfactory bulb (A) to brain stem (K) using *in situ* hybridization with the antisense [^{35}S]-cGKII riboprobe. *In situ* hybridization with the complementary, sense [^{35}S]-cGKII riboprobe (B and L) served as control. Note the strong cGKII expression in the granular and glomerular part of the olfactory bulb (A), the piriform cortex (C–H), hypothalamic and tegmental nuclei (D–F and J), and more moderately in the amygdala (G–I) and parts of thalamus (E–H). Note the broadening of the cGKII mRNA-positive region in the dorsolateral part of the frontal and parietal cortex representing motor and sensory cortex (D–H). Scale bar, 10 mm.

In the light-dark box test, the cGKII $^{-/-}$ mice entered the anxiety-causing light zone six times less frequently than the wild-type animals (Fig. 4). Thus, there was a significant difference in the number of entries to the lit compartment of the light-dark box ($P < 0.001$) (Fig. 4) and the duration to stay in the lit compartment ($P < 0.001$) (Fig. 4). The latency to enter the anxiety-causing zone was also significantly higher for the cGKII $^{-/-}$ mice ($P < 0.001$) (Fig. 4). In the elevated O-maze similar observations were made: cGKII $^{-/-}$ mice had significantly less entries ($P < 0.001$) and spent less time ($P < 0.05$) in the open zone (Fig. 4). The latency to enter the open zone was also significantly higher for the cGKII $^{-/-}$ mice ($P < 0.001$) (Fig. 4). Together these data suggest that cGKII $^{-/-}$ mice are more anxious than their wild-type littermates. In this context, it is important to note that cGKII $^{-/-}$ mice did not differ from control mice in locomotor activity (distance moved in the first 5-min interval, which corresponds to the test time in the anxiety tests: cGKII $^{-/-}$ 845 ± 56 vs. wild-type 806 ± 48 cm), a measure that can interfere with anxiety testing in the light-dark box as well as in the elevated O-maze test.

cGKII knockouts are less sensitive to the hypnotic effects of alcohol and voluntarily consume more alcohol

The cGKII $^{-/-}$ mice of both strains were less sensitive to the hypnotic effects of ethanol as shown in Fig. 5. The onset of the hypnotic effect of ethanol could be seen immediately after the intraperitoneal injection of ethanol. In both groups, the time until loss of righting reflex (LORR) after ethanol injection was similar for the cGKII $^{-/-}$ (122 ± 11 s) and for the wild-type (114 ± 9 s) (values are for the C57BL/6N strain). However, the latency to regain the righting reflex, the criterion for the persistence of the hypnotic ethanol action, was significantly reduced in cGKII $^{-/-}$ mice compared to wild-type controls of the C57/BL/6N strain (Fig. 5A). The cGKII $^{+/+}$ mice regained LORR at 53.6 ± 4.5 min and the group of cGKII $^{-/-}$ mice at 37.5 ± 4.91 min ($P < 0.030$). The same effect was observed when the 129/SvN strain was used. Again, the cGKII $^{-/-}$ mice had a significant reduced sleeping time (90.1 ± 0.1 min vs. 132.0 ± 7.3 min in wild-type littermates ($P < 0.002$) (Fig. 5). The large differences between

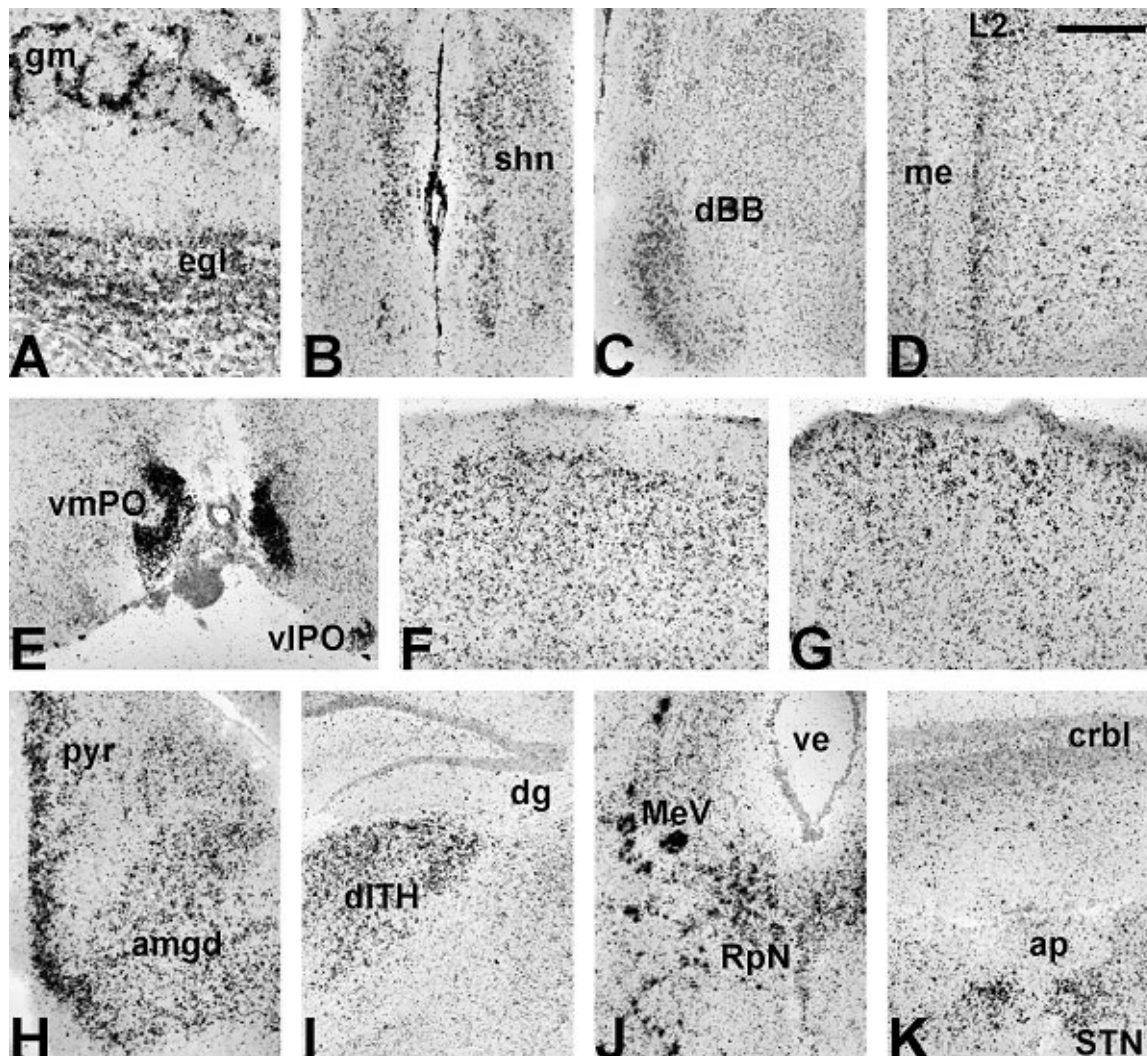


FIG. 2. cGKII mRNA localization in different brain regions at higher magnification, an overview. *In situ* hybridization with antisense [³⁵S]-cGKII riboprobe, autoradiography and haematoxylin/eosin counterstain. (A) olfactory bulb, (B) septum, (C) medial basal forebrain, (D–H) retrosplenial (D), sensory (F), entorhinal (G) and piriform cerebral cortex (H), (E) frontal hypothalamus, (I) dentate gyrus (hippocampus) and dorsolateral thalamic nuclei, (J) periaqueductal midbrain, and (K) lower brainstem and cerebellum. Amgd, amygdala; ap, area postrema; aq, midbrain aqueduct; crbl, cerebellum; dBB, nucleus of the diagonal band of Broca; dg, dentate gyrus; dLTH, dorsolateral thalamic nuclei; egl, external olfactory granule cell region; gm, olfactory glomerula; igl, internal olfactory granule cell region; L2, cerebral cortex layer 2; me, meninges; MeV, mesencephalic trigeminal (V) sensory neurons; mi, mitral cell layer; pyr, pyriform cerebral cortex; Rpn, dorsal midbrain raphe nucleus; shn, septohippocampal nucleus; STN, solitary tract nucleus; viPO, ventrolateral preoptic nucleus; vmPO, ventromedial preoptic nucleus. Scale bar, 0.5 mm.

the measures in wild-type C57BL/6N and 129/SvN need an extra remark; the variation in LORR of different mouse strains is a well-known phenomenon and was also observed for the two strains investigated (Radcliffe *et al.*, 2000).

A possible cause of the shorter hypnotic effect induced by ethanol in cGKII^{-/-} mice might be due to a difference in ethanol clearance compared to wild-type mice. As shown in Fig. 6, no significant difference in ethanol clearance was observed between the cGKII^{-/-} and wild-type mice after the i.p. application of 3 g/kg ethanol. The same result was obtained in C57BL/6N (Fig. 6A) and 129/SvN animals (Fig. 6B). The peak concentration of ethanol measured 0.5 h after application of ethanol was identical for both groups (297 ± 7 and 283 ± 12 mg/dL, for wild-type and cGKII^{-/-} (C57BL/6N strain), respectively). In agreement with this finding, the activity of the liver cytochrome P450 2E1 isoenzyme, assayed as 7-ethoxy-trifluoromethylcoumarin deethylation (Buters *et al.*, 1993) was not different (data not shown). As there is apparently no difference in ethanol

metabolism, the shortened sleeping time in the cGKII mutants presumably results from an altered sensitivity of the central nervous system to hypnotic doses of ethanol.

Mice showed no overall initial side preference when they were offered continuous *ad libitum* access to two bottles of tap water for 1 week. This initial side preference test ensured that subsequent alcohol preference tests were not influenced by an initial side preference. Additionally, the location of the two bottles was changed during the test every three days. In the strain 129/SvN, exposure of the cGKII^{-/-} and wild-type mice to 5% w/v ethanol in a two bottle-free choice-test for 6 days led to a significantly higher ethanol consumption in the knockout mice than in the wild-type controls (Fig. 7A; 3.67 ± 0.69 vs. 2.06 ± 0.45 g/kg/d ethanol, $P < 0.01$; for cGKII^{-/-} $n = 13$ and wild-type $n = 15$, respectively). This effect was reproducible in mice on a homogeneous C57BL/6N background (Fig. 7C; 3.46 ± 0.24 vs. 2.23 ± 0.47 g/kg/d ethanol; $P < 0.01$; for cGKII^{-/-} $n = 10$ and wild-type $n = 12$, respectively). In both strains

TABLE 1. Distribution of cGKII mRNA in the mouse brain

Brain region	cGKII
Telencephalon	
Olfactory bulb	
External plexiform layer	-
Granular layer (GRO), external part	+++
Granular layer (GRO), internal part	-
Periglomerular zones	++++
Mitral cells (MiA)	+++
Cortex (layer II)	
Orbital	++
Frontal	++
Anterior cingulate	+++
Retrosplenial	(+)
Somatosensory/Motor	+++
Entorhinal	+++
Piriform	++++
Visual	+++
Pae-/parasubiculum	(+)
Hippocampus	
CA1 (pyramidal cells)	(+)
Dentate gyrus (granule cells)	-
Basal forebrain	
Amygdala	+++
Septal hippocampal nucleus	+++
Nucleus of the diagonal band of Broca	+++
Islands of Calleja	++
Striatum	+ / ++
Diencephalon	
Thalamus	
Ventrolateral group (LG, MG, VPL, VPCMO)	+++
Anterior dorsomedial group (LPR, LPMR, Po, MDC)	+++
Posterior dorsomedial group	(+)
Habenula	(+)
Hypothalamus	
Nucleus preopticus	++++
Nucleus paraventricularis	+++
Nucleus periventricularis	++
Capsula interna	+
Midbrain	
Substantia nigra	++
Inferior colliculus	+
Raphe nuclei	+++
Dorsal tegmental nuclei	++
Pontine reticular nucleus	+
Hindbrain	
Brainstem	
Nucleus subthalamicus	+
Nucleus spinalis ni. trigemi	++
Nucleus tractus solitarii	+++
Nucleus facialis	+
Vestibular nuclei	-
Cerebellum	
Purkinje cells	-
Granular cells	+
Deep cerebellar nuclei	+

In situ hybridization signals on film and emulsion-dipped sections were related as +, low; (+), very low; -, no signal above background. The brains were obtained from wild-type mice of the C57BL/6N strain.

ethanol preference of cGKII^{-/-} was also significantly enhanced compared to wild-type mice (Fig. 7B and D). Further, the consumption of water was not significantly different between cGKII^{-/-} and wild-type mice in both strains (data not shown). It should be noted that the two strains used in the present study usually exhibit low ethanol intake (Belknap *et al.*, 1993). The finding of elevated ethanol intake in cGKII knockout mice is therefore based on comparisons with two low-drinking background genotypes.

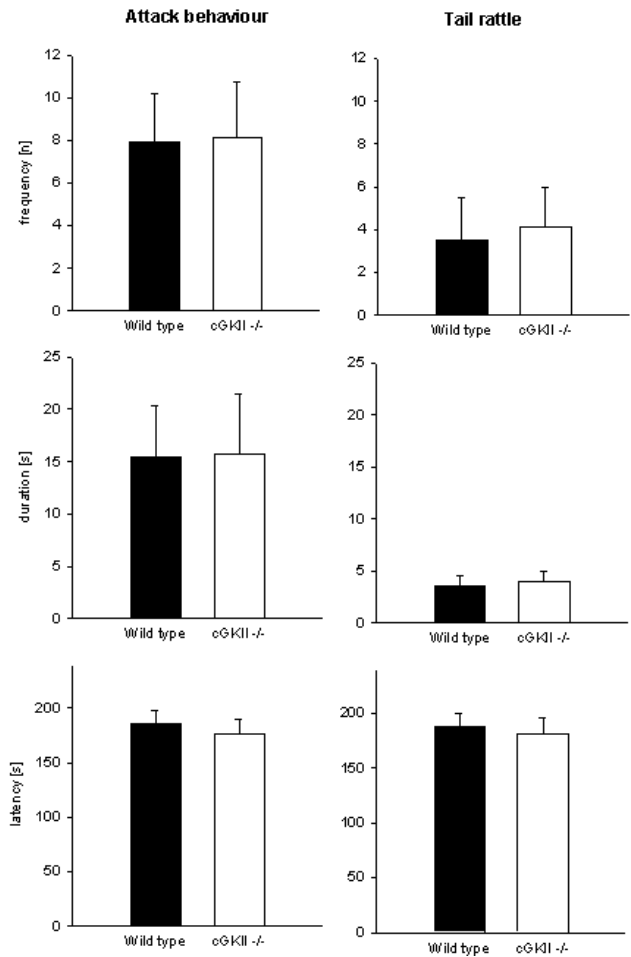


FIG. 3. Measures of aggressive behaviour in the resident/intruder interaction test; frequency, duration and latency of attack behaviour and tail rattles by wild-type ($n = 15$) and cGKII^{-/-} knockout mice ($n = 14$). All measures are given as mean \pm SEM. There were no significant differences between genotypes for these two measures.

In rodents, alcohol intake is partially dependent upon its flavour. Thus, alcohol taste has a sweet-bitter component and the proclivity to drink alcohol is associated with elevated sweet preferences and/or lower aversion to a bitter taste. The sweet taste component of alcohol that occurs at concentrations up to 6% can thus enhance alcohol preference (Spanagel *et al.*, 2002). We therefore performed taste preference experiments with sucrose and saccharin. Compared to their wild-type controls, the cGKII^{-/-} mice did not show an increased preference towards 4% sucrose or 0.06% saccharin containing water (Table 2). Under any condition, the total intake of fluid was not different between the wild-type and cGKII-knockout mice (data not shown).

Discussion

The data presented show that cGKII is widely expressed in the mouse brain. Strong expression was noted in the olfactory bulb, parts of thalamus and cerebral cortex, basal forebrain and periventricular hypothalamic nuclei, and some sensory brainstem nuclei such as the solitary tract nucleus and the spinal nuclei of the trigeminal nerve. Based on these neuroanatomical data, a deficiency in cGKII could result in a variety of behavioural and sensory deficits, e.g. in olfaction

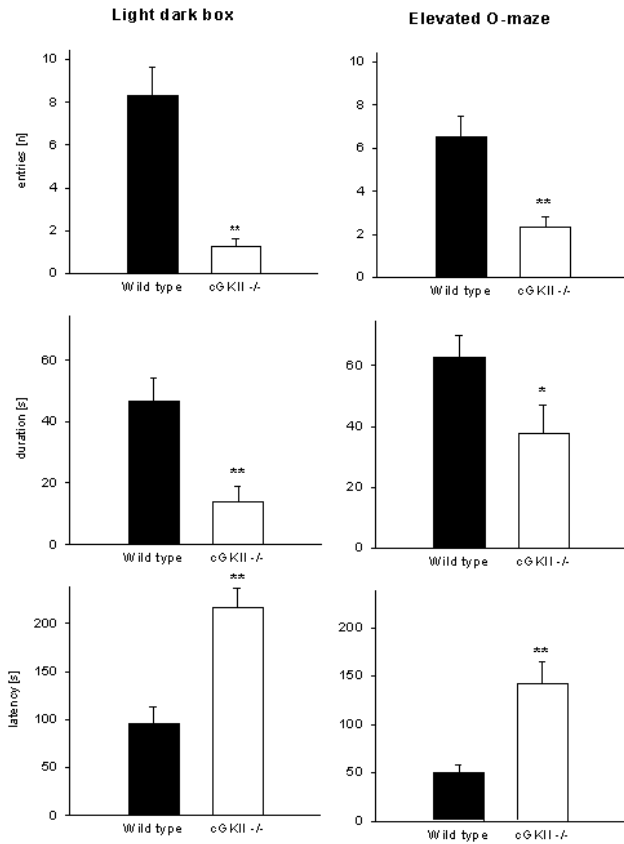


FIG. 4. Measures of anxiety-like behaviour in the light-dark box test and elevated O-maze test; number of entries, duration of time spent either in the lit compartment or the open zone of the elevated O-maze, and latency to enter the anxiety causing zone by wild-type ($n = 20$) and cGKII^{-/-} knockout mice ($n = 19$). All measures are given as means \pm SEM. * $P < 0.05$ ** $P < 0.001$.

(olfactory bulb, diagonal band of Broca), hearing (thalamic medial geniculate nucleus), vision (lateral geniculate nucleus, visual cortex), taste (solitary tract nucleus) or touch and perception of vibrissae movement (spinal nucleus of the trigeminal nerve). The cGKII expression in the preoptic and periventricular hypothalamic nuclei may be involved in endocrine and circadian rhythm functions, that in septal nuclei and upper neuronal layer of the cerebral cortex in generalized cortical function and the susceptibility to sleep-inducing substances. In the current study, we have concentrated on the effects of cGKII deletion on the sleep-inducing and addictive effects of ethanol, anxiety and the aggressive behaviour.

The relatively high levels of cGKII in the basal forebrain, including the amygdala, suggest that this enzyme might have a role in mediating behavioural functions and emotional responses. Indeed, cGKII^{-/-} mice do show increased anxiety-like behaviour without altered locomotor activity. Furthermore these mice are less sensitive to the hypnotic effects of ethanol and voluntarily consume more alcohol compared to wild-type littermates. In summary, our data show that cGKII is involved, at least in part, in controlling anxiety-like behaviour and regulating the neurobehavioural responses to alcohol.

cGMP regulates a variety of enzymes and proteins. However, the vast majority of the biological actions of cGMP can be attributed to the activation of a specific cGMP-dependent protein kinase. Two major isoforms of the vertebrate enzyme have been identified, a cytosolic type I (cGKI) and a membrane-bound type II (cGKII) (Hofmann *et al.*, 2000). Although cGKI is distributed fairly widely in mammalian cells, its expression in the brain is restricted to just a few

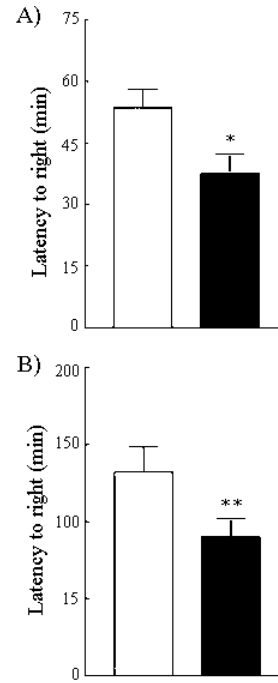


FIG. 5. Sensitivity to the hypnotic effect of ethanol assessed by the measurement of loss of righting reflex (LORR) in wild-type and cGKII^{-/-} mice. In Panel A and B, C57BL/6N ($n = 22$) and 129/SvN strains were used, respectively. Ethanol (4 g/kg) was injected i.p. and the time to regain the righting effect was measured. Each value is the mean \pm SEM. Asterisks indicate a significant difference from wild-type littermate mice (* $P < 0.030$ and ** $P < 0.002$).

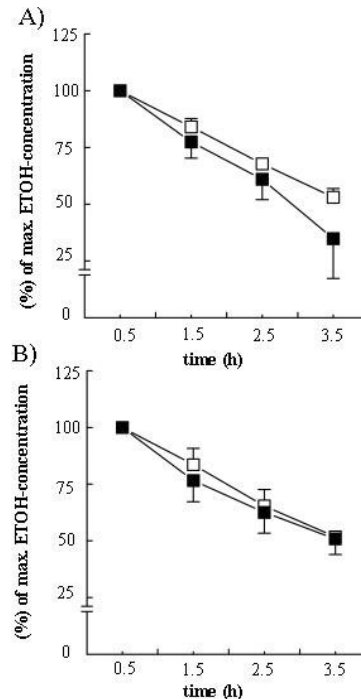


FIG. 6. Ethanol clearance of cGKII-deficient (cGKII^{-/-}, $n = 6$, filled squares) and wild-type mice ($n = 6$, open squares) after i.p. application of 3 g/kg ethanol (20%, w/v). In Panel A and B, C57BL/6N and 129/SvN strains were used, respectively. Each value is the mean \pm SEM. No significant difference between genotypes for ethanol elimination could be detected.

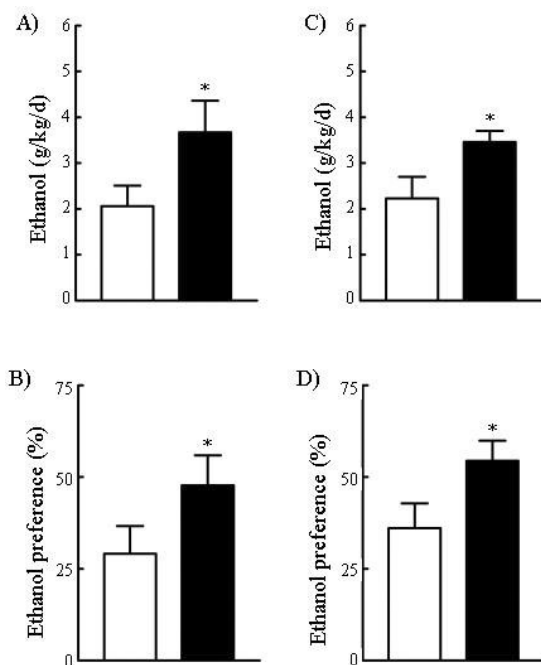


FIG. 7. Ethanol intake (A and C) and ethanol preference (B and D) in cGKII^{-/-} mice (black bar) and wild-type littermates (closed bars) of the 129/SvN (A and B) and C57BL/6 N (C and D) strain. The number of animals was 28 and 22 in the 129/SvN and C57BL/6N groups for each genotype, respectively. Each value is the mean \pm SEM. * $P < 0.01$, compared with wild-type littermate mice.

TABLE 2. Voluntary consumption of sweet solutions

Drinking solution	Consumption (mL/day)	
	Wild-type littermates	cGKII ^{-/-}
Sucrose (4%)	13.5 \pm 1.4	14.4 \pm 1.7
Saccharine (0.06%)	7.0 \pm 0.98	9.1 \pm 1.3

Wild-type and knockout (cGKII^{-/-}) mice ($n = 19$ for each genotype) of the C57BL/6N strain were exposed on six consecutive days to a two-bottle procedure with either water and 4% sucrose, or water and 0.06% saccharin. Compounds were dissolved in water. No significant differences could be detected.

brain sites, with the most abundant levels in Purkinje cells. In contrast, cGKII has a much wider distribution in the rat brain (El-Husseini *et al.*, 1995; De Vente *et al.*, 2001). Our present *in situ* hybridization studies extend these earlier findings obtained in the rat brain showing that high levels of cGKII mRNA occur in parts of the mouse cortex, thalamus and basal forebrain structures including the amygdala and striatum.

So far, it is not clear which physiological functions are mediated by NO/cGMP/cGKII signalling. Here, we provide the first evidence that anxiety-like behaviour is, at least in part, controlled by this intracellular signalling pathway. This finding is not confounded by changes in locomotor activity as cGKII mice show the same locomotor response to novelty as wild-type mice. Aggressive behaviour, which has been also attributed to NO/cGMP signalling, was unchanged in cGKII deficient mice. This result is somewhat surprising as it has been reported that nNOS knockout mice display a high level of aggressive behaviour (Nelson *et al.*, 1995; Chiavegatto *et al.*, 2001). Thus it seems to be that aggressive behaviour is controlled by

NO/cGMP signalling through either cGMP-gated ion channels or cGMP-regulated phosphodiesterases but not through cGKII. In summary, anxiety-like behaviour is controlled through NO/cGMP/cGKII signalling, a finding that corresponds to the expression levels of cGKII in brain sites thought to be involved in emotionality.

Enhanced anxiety in rodents is often associated with enhanced alcohol consumption although this relationship is complex (Spanagel & Höltter, 1999). Indeed, cGKII deficient mice voluntarily consume more alcohol than wild-type littermates. This finding has been replicated several times in our laboratory in different strains (129/Sv and C57BL/6N), making it unlikely that genetic background problems or environmental factors are interfering with this phenotype (Gerlai, 1996; Crabbe *et al.*, 1999; Wahlsten *et al.*, 2003). In agreement with this finding, the cGKII^{-/-} mice were more resistant to the hypnotic effects of ethanol than the wild-type animals. The discrete differences between the two groups of animals can not be explained by altered ethanol metabolism or preference differences for sweet tasting beverages. Both strains harbouring the gene deletion showed the same ethanol elimination rates than control mice. Moreover, the activity of the liver cytochrome P450 2E1 isoenzyme was not different in these animals. Taste experiments also did not yield any differences. In this context, it should be noted that alcohol intake in mice is partially dependent on its flavour. Thus, alcohol drinking is often associated with elevated sweet preferences and/or lower aversion to a bitter taste (Bachmanov *et al.*, 1996). However, in our taste preference experiments no differences in intake of sweet solutions could be observed, making it unlikely that a sweet-like taste component of a 5% ethanol solution (Li *et al.*, 2001) might have interfered with enhanced alcohol consumption in cGKII deficient mice.

Little is known about the involvement of NO/cGMP signalling via cGKII in mediating behavioural responses in mammals, although cGKII affects the phase shifts of the circadian clock (Oster *et al.*, 2003). There is ample evidence that this pathway is important in mediating a variety of behaviours in invertebrates. In flies, bees and *C. elegans*, cGMP kinase is essential for different aspects of food searching strategies (Renger *et al.*, 1999; Schafer, 2002). In fly larvae, cGMP kinase is inactivated in the sitters (Osborne *et al.*, 1997), whereas in bees the enzyme is expressed when the bee changes from a worker bee to a nectar collecting individual (Ben-Shahar *et al.*, 2002). In *C. elegans*, cGMP kinase is expressed in chemosensory (AWC) neurons and is necessary for the adaptation of odour sensing AWC neurons (L'Etoile *et al.*, 2002). Further, cGMP-dependent kinase functions in neurons of *C. elegans* to regulate multiple developmental and behavioural processes (Fujiwara *et al.*, 2002). In summary, these findings suggest that cGMP kinase is involved in several vital behaviours in a wide range of animals and here we extend these findings by showing that emotionality and drug responsiveness also depends on cGMP kinase activity in mammals.

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Abbreviations

cGKII, cGMP-dependent protein kinase type II; LORR, loss of righting reflex; nNOS, neuronal NO synthase; NO, nitric oxide.

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