

Adenosine Receptor Antagonists Including Caffeine Alter Fetal Brain Development in Mice

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Consumption of certain substances during pregnancy can interfere with brain development, leading to deleterious long-term neurological and cognitive impairments in offspring. To test whether modulators of adenosine receptors affect neural development, we exposed mouse dams to a subtype-selective adenosine type 2A receptor (A_{2A}R) antagonist or to caffeine, a naturally occurring adenosine receptor antagonist, during pregnancy and lactation. We observed delayed migration and insertion of γ -aminobutyric acid (GABA) neurons into the hippocampal circuitry during the first postnatal week in offspring of dams treated with the A_{2A}R antagonist or caffeine. This was associated with increased neuronal network excitability and increased susceptibility to seizures in response to a seizure-inducing agent. Adult offspring of mouse dams exposed to A_{2A}R antagonists during pregnancy and lactation displayed loss of hippocampal GABA neurons and some cognitive deficits. These results demonstrate that exposure to A_{2A}R antagonists including caffeine during pregnancy and lactation in rodents may have adverse effects on the neural development of their offspring.

INTRODUCTION

Neural development is strongly influenced by external (for example, environmental) factors including certain drugs (1). These drugs are known to modify cell migration (2, 3) and synapse formation (4, 5), resulting in improper wiring of neuronal circuits, ultimately leading to behavioral modifications in offspring later in life (6). There have been few studies about the effects on fetal neural development of other substances such as the naturally occurring adenosine receptor antagonist caffeine, which is ubiquitously consumed in coffee and tea. Mild to moderate caffeine consumption (<200 mg/day) by mothers during pregnancy and lactation is not considered harmful to the fetus and neonate from the perspective of miscarriage or risk of prematurity. This is reflected in the current guidelines for caffeine consumption during pregnancy by the American College of Obstetricians and Gynecologists (7–9). Although caffeine can cross the placenta and blood-brain barrier (7), its impact on fetal and early postnatal development remains inconclusive (10). Caffeine has several molecular targets. At high concentrations (millimolar range), caffeine can inhibit phosphodiesterases resulting in mobilization of calcium stores or it may have a direct effect on γ -aminobutyric acid type A (GABA_A) receptors (11). Such high concentrations, considered toxic, are rarely found in the blood of consum-

ers of mild to moderate amounts of caffeine (7). In the micromolar range, a concentration regularly found in humans (7), caffeine acts as an antagonist of adenosine receptors, which modify numerous intracellular signaling pathways including cyclic adenosine monophosphate (cAMP)-dependent signaling (12). We set out to investigate the effects of caffeine consumption by pregnant rodent dams on brain development in their offspring. Because caffeine absorption takes place in the gastrointestinal tract (7), we used a protocol of oral caffeine administration in mouse dams throughout pregnancy and lactation. We investigated the short-term (during the first postnatal week) and long-term (during adulthood) consequences of exposure to caffeine or a subtype-selective adenosine type 2A receptor (A_{2A}R) antagonist during fetal development in the dam's offspring.

RESULTS

General effects of caffeine treatment on female mouse dams and their pups

To mimic a routine daily consumption in human subjects, we exposed female mice to caffeine (0.3 g/liter) in their drinking water during pregnancy and lactation. This dose (0.3 g/liter) leads to a plasma concentration of caffeine in rat dams similar to that found in the blood of humans drinking three to four cups of coffee per day (13) and to a plasma concentration of caffeine in postnatal day 7 (P7) rat pups similar to that found in the umbilical cord of human neonates of moderate coffee-drinking (up to three cups per day) mothers (14). We found a similar concentration of caffeine in female mice that were exposed to caffeine (0.3 g/liter) in their drinking water [caffeine (1.0 \pm 0.7 mg/liter) in serum]. Treated females (n = 7) adapted quickly to the addition of caffeine to the drinking water and thus showed no difference in water intake from 2 days after the start of caffeine treatment onward (fig. S1A). Food intake of caffeine-treated and untreated female mice was similar

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(untreated, 3.48 ± 0.12 g/day; treated, 3.48 ± 0.13 g/day; $P = 1.0$). There was no difference in weight increase throughout pregnancy between control and caffeine-treated dams (fig. S1B).

Addition of caffeine to the drinking water led to enhanced activity during the active (dark) period, but not during the sleep (light) period of the day. The day-night cycle was not affected by the treatment (fig. S1C). During the first 2 weeks of life, the general condition of pups was qualitatively assessed during the dark period every second day. In both caffeine-treated and control groups, all dams built a nest and pups were in all cases found with the mother within the nest. The gestational period of the control and caffeine-treated females was similar (19 to 20 days), and the litter sizes were not different between the two groups (fig. S1D). Caffeine was present in the brain of pups, where it reached concentrations of 1.06 ± 0.19 ng/mg together with its metabolic products, namely, theobromine (0.62 ± 0.02 ng/mg) and 1,3-dimethyluric acid (0.21 ± 0.04 ng/mg) ($n = 5$, P1 to P8 pups). Pups born from caffeine-treated dams displayed a transient body weight reduction between P1 and P11 that was gender-independent and that was normalized at P24 (fig. S1E). The weight of 13-week-old adult offspring was not changed by caffeine exposure (caffeine-treated group, 22.2 ± 3.5 g; control group, 24.2 ± 3.2 g; $P = 0.22$). Motor development and development of neurological reflexes during the first 11 days of life were not affected by caffeine exposure because the righting reflex (fig. S1F) and cliff avoidance behavior (fig. S1G) were not significantly different between caffeine-exposed and control offspring.

Together, these data indicate that caffeine treatment during pregnancy and lactation has a major influence neither on female mice nor on pups' sensorimotor development. We next assessed whether the construction of neuronal networks was affected by caffeine treatment, focusing on GABAergic circuits, which are often altered by various types of insults.

Caffeine treatment of dams alters GABA neuron migration in their offspring

Using GIN mice [green fluorescent protein (GFP)–expressing inhibitory neurons] that express GFP in a subpopulation of somatostatin-containing GABA neurons (15, 16), we found a consistent decrease in GFP-labeled neurons in the hippocampus and superficial cortical layers of all caffeine-exposed offspring ($n = 11$ pups) compared to controls ($n = 10$ pups) at P6 (Fig. 1, A and B). There was a 41% ($P < 0.05$) decrease in GABA neurons in the hippocampus of caffeine-exposed offspring at P6 (Fig. 1H and Table 1). In contrast, the distribution pattern (Fig. 1, D and E) and estimated number (Fig. 1H and Table 1) of hippocampal GFP-expressing neurons were similar in 3-month-old adult control and caffeine-exposed offspring. We thus hypothesized that caffeine may delay the migration of GABA neurons.

Cortical GABA neurons are generated within the ventral telencephalon by the medial and caudal ganglionic eminences (MGE and CGE) in mice (17–19); somatostatin-containing neurons are generated in the MGE (20, 21). Cortical GABA neurons first migrate tangentially before switching to a radial migration as they invade the cortical plate. Many GABA neurons keep migrating postnatally to reach their target layers (18, 22, 23). In keeping with this rule of migration, in control GIN mice, some GFP-labeled neurons were observed in the deep cortical layers at P1 (fig. S2, A and E), as they started their radial migration to the superficial layers. At P3, few GFP-labeled neurons were positioned in the superficial supragranular layer (fig. S2, B and F) and in the stratum oriens of the hippocampus (fig. S2I). At P6, many mor-

phologically differentiated GFP-labeled neurons were evident in the superficial cortical layers (fig. S2, D and H) and in the hippocampus (Fig. 1A and fig. S2K). The distribution patterns of GFP-labeled neurons and axon terminals in caffeine-exposed offspring at P6 (fig. S2, C, G, and J) were similar to that observed at P3 in control mice (fig. S2, B, F, and I), supporting the hypothesis that caffeine delayed the migration and insertion of interneurons in cortical networks.

Caffeine treatment affected the migration of the entire population of somatostatin-containing GABA neurons (fig. S3, A to F). Somatostatin-containing neurons displayed an immature distribution pattern in caffeine-exposed offspring at P6, illustrated by the presence of many somatostatin-containing neurons, including migrating neurons in the stratum radiatum (control, 25 neurons/mm², $n = 3$ pups from three dams; caffeine-exposed, 58 neurons/mm², $n = 3$ pups from three dams) similar to that observed at an earlier developmental stage in control mice (fig. S3, A to F).

To assess the general state of the GABAergic circuitry, we investigated the distribution pattern of the vesicular GABA transporter (VGAT), which labels GABAergic terminals. VGAT labeling displayed immature features in caffeine-exposed offspring at P6, namely, low concentrations of labeled terminals in the CA3-CA1 pyramidal cell (PC) layer and high concentrations in dendritic layers, as found in control P3 animals (fig. S3, G and H) (24). In contrast, control P6 mice displayed a high concentration of terminal labeling in the PC layer typical of more mature networks (fig. S3I).

The delayed insertion of GABA neurons in cortical circuits suggests that caffeine may interfere with processes that control GABA neuron migration. This hypothesis was directly tested in vitro using time-lapse video microscopy (Fig. 1I). In cocultures prepared at embryonic stage 13.5 (E13.5), GABA neurons from the MGE migrate radially away from their explants of origin on a substrate of dissociated cortical cells (25, 26). Thirty to 45 min after caffeine application, the mean migration speed of MGE neurons was decreased by 53% ($P < 0.001$, $n = 46$ of 52 cells), an effect that persisted until the end of the recording session, up to 10 hours in some experiments (Fig. 1, J and K). A small percentage of MGE cells (11.5%, $n = 6$ of 52 cells) did not respond to caffeine application or responded by increasing their migration speed. We then investigated the adenosine receptor subtype(s) involved in the control of GABA neuron migration.

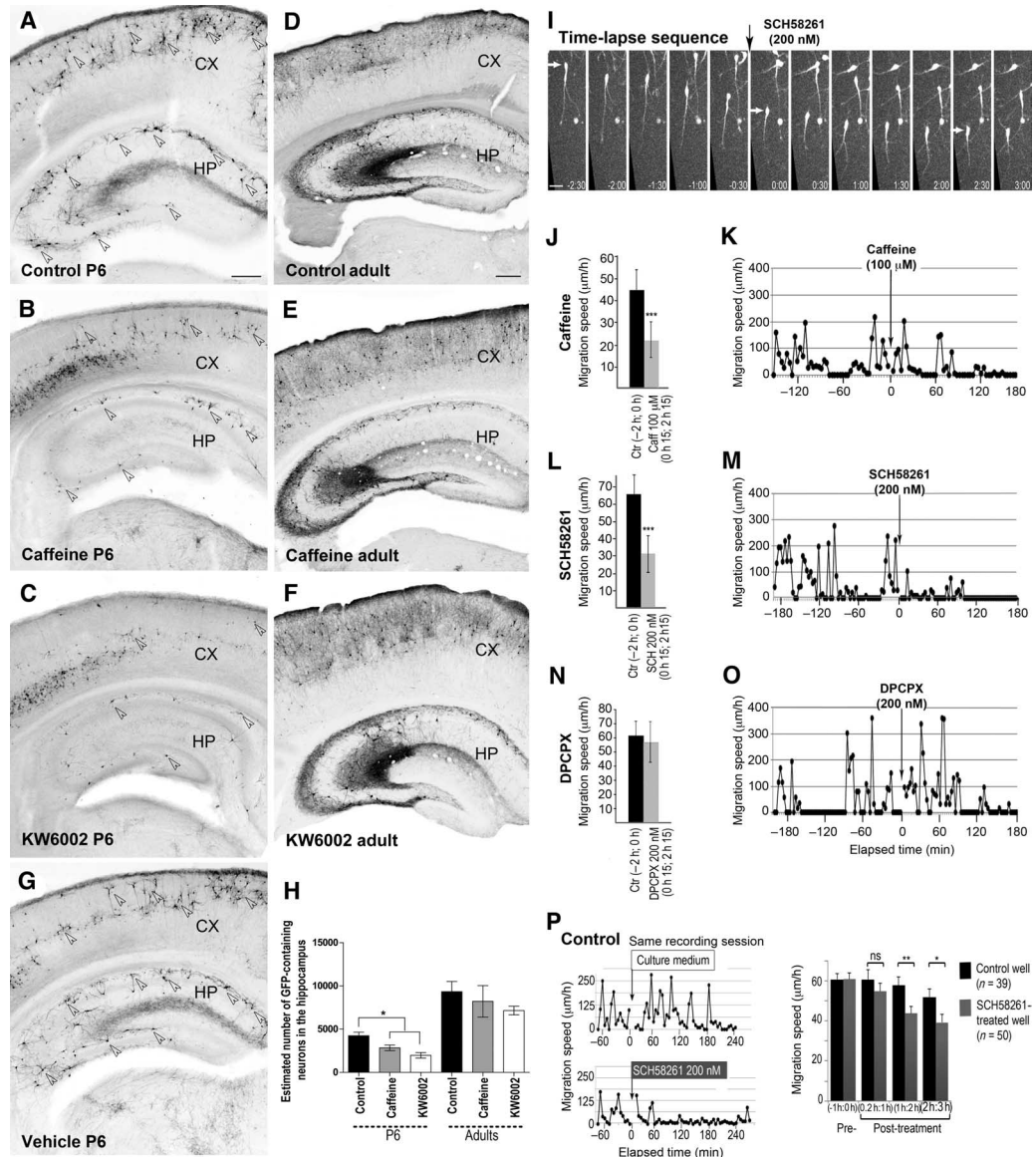
A_{2A}Rs may control the migration of GABA neurons

Chronic consumption of caffeine exerts its central action mostly via its antagonistic action on A_{2A}Rs (27, 28). We thus substituted caffeine with the selective A_{2A}R antagonist KW6002 (istradefylline, 2 mg/kg per day). Exposure to KW6002 (Fig. 1, C, F, and H), but not to its vehicle solution (Fig. 1G), reproduced the effects of caffeine, suggesting that caffeine delayed GABA neuron migration via its antagonistic action on A_{2A}R (Table 1). Application of the selective A_{2A}R antagonist SCH58261 (200 nM) to MGE neurons reproduced the effect of caffeine, reducing the migration speed of MGE cells by 56% ($P < 0.001$, $n = 57$ of 62 cells), with a small percentage of cells (8%, $n = 5$ of 62) not responding to the antagonist or increasing their migration speed (Fig. 1, I, L, and M). In contrast, the A₁ receptor antagonist DPCPX (200 nM) did not change the migration speed of MGE cells (Fig. 1, N and O). In the absence of drug, the migration speed of MGE neurons was not modified throughout the recording period (Fig. 1P).

These results demonstrate that the endogenous activity of A_{2A}R is important for GABA neuron migration. We thus determined whether A_{2A}Rs are indeed expressed by migrating GABA neurons. Using immu-

Fig. 1. Caffeine exposure during the embryonic and early postnatal period impairs the migration of hippocampal GABA neuron subpopulations in mouse brain. (A to F)

Representative coronal sections (6 to 10 examined per animal) from control (A and D), caffeine-exposed (B and E), and KW6002-exposed (C and F) offspring of GIN mice at postnatal day 6 (P6) (A to C) and at 3 months (adult) (D to F). Photomicrographs show the distribution pattern of GFP-containing neurons (white arrowheads) in the hippocampus (HP) and cortex (CX). (A) Many GFP-containing neurons were already present in the superficial layers of the cortex and hippocampus in control pups. (B and C) Caffeine or KW6002 exposure during gestation resulted in a marked decrease in GFP-labeled cell bodies (white arrowheads), axons, and dendrites in the hippocampus and cortex at P6. In contrast, no major differences in GFP labeling of the hippocampus and cortex were observed between control (D) and caffeine-exposed (E) or KW6002-exposed (F) adult offspring. (G) Exposure to vehicle during gestation did not modify the pattern of distribution of GFP-containing neurons in offspring. (H) Estimated total number of GFP-expressing neurons in the hippocampus of caffeine-exposed ($n = 3$) or KW6002-exposed ($n = 2$) P6 offspring was smaller ($P < 0.05$) than in control ($n = 4$). In contrast, the estimated number of GFP-containing neurons was similar in the three adult groups ($n = 3, 3$, and 4, respectively; $P = 0.508$). (I) Representative time-lapse video sequence illustrating a GFP-expressing MGE mouse neuron (white arrow) in vitro migrating on a substrate of dissociated cortical cells in response to the application of SCH58261 [5-amino-7-(2-phenylethyl)-2-(2-furyl)pyrazolo(4,3-e)-1,2,4-triazolo(1,5-c)pyrimidine] (vertical black arrow) (time is in hours:minutes). (J to O) Caffeine or SCH58261, but not DPCPX (8-cyclopentyl-1,3-dipropylxanthine), decreased the migration speed of MGE cells in coculture. (J, L, and N) Bar graphs compare the mean migration speed of more than 50 MGE cells measured during a 2-hour period before and after application of caffeine (J, $n = 52$), SCH58261 (L, $n = 62$), or DPCPX (N, $n = 83$). $P < 0.001$ in (J) and (L), not significant in (N), Student's t test. (K, M, and O) Time course of instantaneous migration speed for repre-



sentative MGE cells exposed to caffeine (K), SCH58261 (M), or DPCPX (O). Vertical black arrow indicates when the drug was applied. (P) In the absence of drug, the mean migration speed of MGE cells did not change during the recording period as demonstrated by the curves on the left, comparing the time course of nuclear movements in two MGE cells recorded in two contiguous cocultures: one without treatment (top) and one treated with SCH58261 (bottom). Right: The decrease in the mean nuclear migration speed was significant 1 hour after SCH58261 application (gray bars). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Scale bars, 250 μm (A to G) and 20 μm (I).

nohistochemistry, we found $A_{2A}R$ in somatostatin-containing GABA neurons, leaving the ganglionic eminence at E13 in rats, in particular within the leading process, a key element of cell migration (29) (Fig. 2, A to F). A similar scenario may be at play in primates as we also found $A_{2A}R$ in tangentially and pseudo-radially migrating GABA neurons (30) in macaque fetal brain at E75 (Fig. 2, G and H). The intracellular pathways linking $A_{2A}R$ activation and cell motility, as well as the source of adenosine remain to be determined.

To further confirm that caffeine interferes with $A_{2A}R$ -dependent migration of somatostatin-containing GABA neurons, we performed caffeine and KW6002 treatments in mouse dams constitutively lacking $A_{2A}R$ ($A_{2A}R$ -KO). All $A_{2A}R$ -KO pups showed a decreased number of somatostatin-containing GABA neurons in both the hippocampus and superficial cortical layers (fig. S4, A to F). Somatostatin-containing neurons of $A_{2A}R$ -KO pups displayed poorly developed dendritic profiles compared to wild-type pups of the same strain (fig. S4, G and H),

Table 1. Estimated number of GFP-containing neurons in control, caffeine-exposed, and KW6002-exposed pup and adult offspring.

The number of GFP-containing neurons was decreased in the hippocampus of caffeine- and KW6002-exposed P6 offspring but not adult

offspring. Given that the morphological pattern of distribution, as well as the immunoreactivity, in hippocampus from vehicle-exposed animals was similar to controls, a stereological quantification was not performed in the vehicle group.

	P6 control	P6 caffeine	P6 KW6002	Adult control	Adult caffeine	Adult vehicle
Experimental number	4 pups, 3 dams	3 pups, 3 dams	2 pups, 3 dams	4	3	3
Estimated number of GFP-containing neurons	4239 ± 398	2838 ± 317*	1971 ± 315 ^{††}	9336 ± 1173	8213 ± 1825	7152 ± 500

**P* < 0.05 versus control, one-way analysis of variance (ANOVA).

^{††}*P* < 0.05 versus caffeine, one-way ANOVA.

suggesting that loss of A_{2A}Rs impairs the migration and insertion of GABA neurons in cortical and hippocampal layers. Neither caffeine nor KW6002 treatment further decreased the number of GABA neurons in the hippocampus of A_{2A}R-KO pups (fig. S4I), further supporting the notion that the phenotypic traits found in caffeine or KW6002-treated wild-type animals are due to a selective action of these drugs on A_{2A}Rs.

Abnormal GABA neuron migration may lead to altered network function (31). We thus assessed whether exposing dams to caffeine modified electrophysiological activity in the hippocampus of their offspring.

The hippocampus is hyperexcitable in caffeine-treated rodent offspring

During the first postnatal week, in vitro hippocampal activity is characterized by the occurrence of giant depolarizing potentials (GDPs), which constitute a good readout of network activity and excitability (32). The frequency of GDPs in the hippocampal CA3 region was 217% higher in caffeine-treated pups compared to controls and 206% higher in KW6002-treated compared to vehicle-treated pups (Fig. 3, A and B, and table S1). The properties of GDPs depend on the activation of glutamate and GABA_A receptors (32). Measuring both GABAergic and glutamatergic synaptic inputs (drives) received by CA3 PCs during GDPs, we found a 74% increased contribution of the glutamatergic synaptic input to the overall synaptic input (glutamate + GABA) (Fig. 3C and table S2) in caffeine-exposed animals (*n* = 7 cells from five pups, four dams) compared to controls (*n* = 6 cells from three pups, three dams) and a 130% increase in KW6002-treated pups (*n* = 3 cells from three pups, three dams) compared to vehicle-exposed pups (*n* = 5 cells from three pups, two dams). In contrast, in stratum radiatum interneurons, the glutamatergic synaptic input was similar in control (*n* = 10 cells from three pups, two dams), vehicle-exposed (*n* = 6 cells from three pups, two dams), caffeine-exposed (*n* = 15 cells from four pups, four dams), and KW6002-exposed (*n* = 14 cells from four pups, four dams) animals (Fig. 3C and table S2).

In CA3 PCs, the frequency of spontaneous inhibitory postsynaptic currents (sIPSCs) and spontaneous excitatory postsynaptic currents (sEPSCs), which are measured between GDPs, was increased by 238 and 466%, respectively, in caffeine-exposed, and by 241 and 350%, respectively, in KW6002-exposed pups (Fig. 3, D to G, and table S3). In stratum radiatum interneurons, the frequency of sIPSCs and sEPSCs was increased by 428 and 325%, respectively, in caffeine-exposed, and by 407 and 245%, respectively, in KW6002-exposed animals (Fig. 3, D to G, and table S3). The corresponding decrease in the mean interevent interval (IEI), which is the time between two consecutive synaptic inputs, is displayed in the histograms below the frequency curves. The contribution of the glutamatergic synaptic input carried by sEPSCs to

the overall synaptic input in CA3 PCs was increased by 134% in caffeine-exposed and 125% in KW6002-exposed pups (Fig. 3H and table S4). However, it remained unchanged in stratum radiatum interneurons (Fig. 3H and table S4) because both glutamatergic and GABAergic drives were similarly increased in caffeine- or KW6002-exposed animals. There is thus a general increased synaptic (glutamatergic and GABAergic) and network (GDP) activity in caffeine-exposed offspring compared to controls. Although appearing counterintuitive given the delayed insertion of GABA neurons, an increase in GABAergic activity is consistently found in several pathological contexts. For example, mice lacking lissencephaly-1 protein (33, 34) are characterized by defects in GABA neuron migration (35), which are overcompensated for by hyperactivity of the remaining GABA neurons (33, 34).

Network hyperactivity can induce neuronal injury, in particular in GABA neurons (36), and is typified by dendritic swellings. In keeping with the increased network activity (GDPs, sEPSCs, and sIPSCs) received by GABA neurons, we consistently found large varicosities (suggesting swelling) along the dendrites of all GFP-expressing neurons in GIN mice (Fig. 3J) and recorded biocytin-filled interneurons (Fig. 3L) in caffeine-exposed offspring at P6. These varicosities were not observed in interneurons in controls (Fig. 3, I and K) or in PCs from caffeine-exposed offspring (Fig. 3M). Such selective effects on GABA neurons may result in cell dysfunction, perhaps contributing to the shift in the balance between excitatory and inhibitory inputs in principal cells.

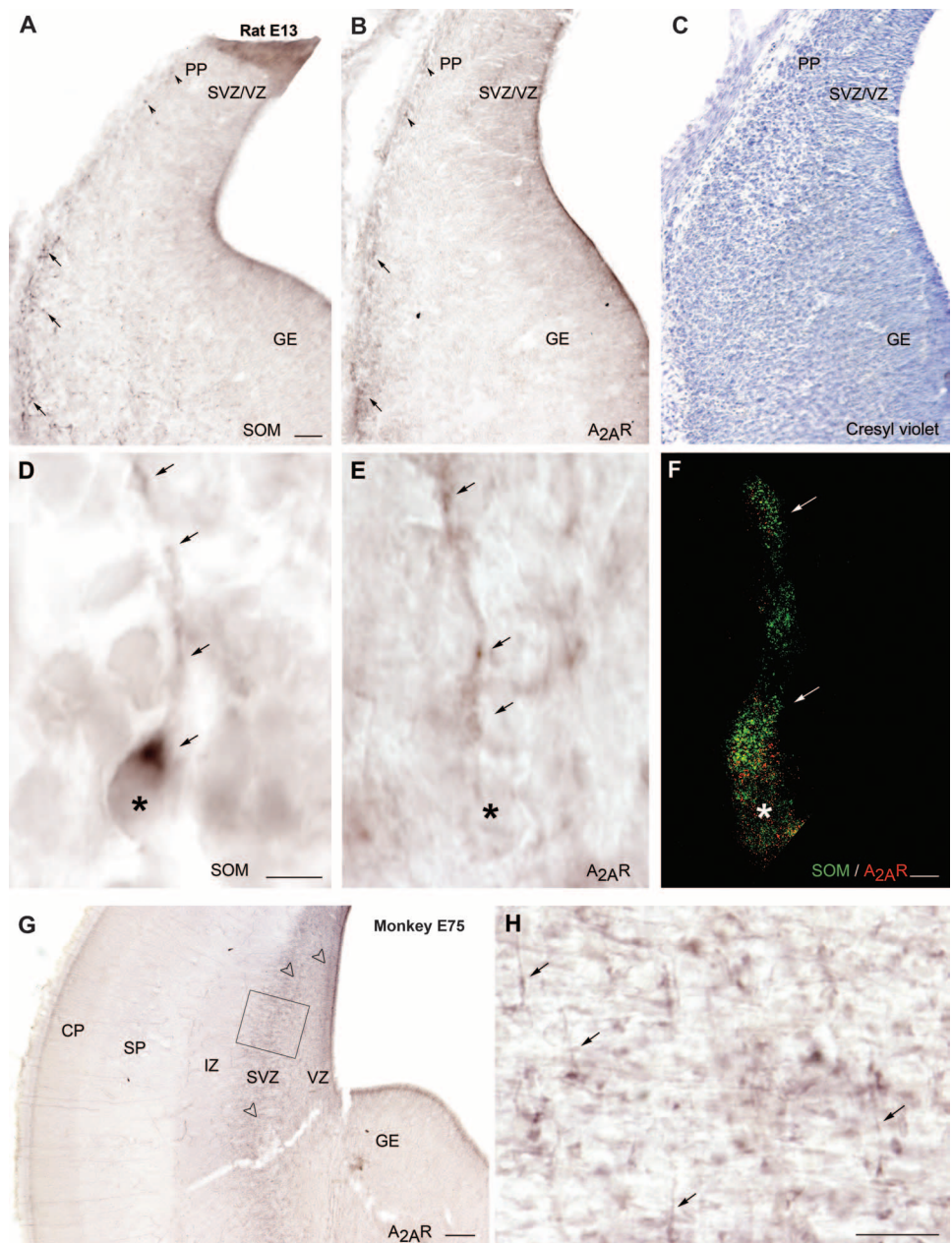
We conclude that the anatomical alterations induced by caffeine (or KW6002) exposure are paralleled by physiological modifications in the developing brain of offspring. The hippocampal circuitry is characterized by a general hyperactivity, as evidenced by an increase in the frequency of GDPs, sEPSCs, and sIPSCs, as well as a shift in the balance between the GABAergic and glutamatergic synaptic inputs in favor of the latter in CA3 PCs.

Immature neuronal networks are seizure-prone (37), and defects in GABA neuron migration or in GABAergic signaling during development facilitate the emergence of an epileptic phenotype (31, 38). We thus tested seizure sensitivity in caffeine-exposed offspring during the first postnatal week.

Caffeine exposure increases susceptibility of mouse pups to seizures

We used flurothyl, a seizure-inducing agent (39), to assess seizure susceptibility in caffeine-exposed and control offspring at P6. Pairs of caffeine-exposed/control and KW6002-exposed/control pups at P6 were exposed to flurothyl in two independent sessions (four treated and four control animals per session). None of the pups analyzed (eight controls from two dams, four caffeine-exposed pups from one dam, and four KW6002-exposed pups from one dam) displayed seizures when

Fig. 2. A_{2A}Rs are expressed by migrating GABA neurons in the rat and monkey fetal telencephalon. (A to C) Representative adjacent coronal sections (of five to six examined per animal) of the rat telencephalon at E13. (A) Most newly generated somatostatin-containing neurons (SOM; black arrows) were located in the preplate (PP) of the ventral telencephalon, at the level of the ganglionic eminence (GE) with a few in the preplate of the adjacent dorsal telencephalon (black arrowheads). (B) Immunolabeling of A_{2A}Rs was detected in these same regions (arrows). (C) Cresyl violet-stained section showing the ganglionic eminence and the proliferative zone of the dorsal telencephalon including the subventricular (SVZ) and ventricular (VZ) zones. (D) Higher magnification of a somatostatin-containing neuron from (A) with a characteristic morphology of a migrating neuron including a leading process (black arrows). (E) Accumulation of A_{2A}R immunolabeling in the cell body and leading process (black arrows) of a migrating somatostatin-containing neuron. (F) Confocal image of a migrating somatostatin-containing neuron in the preplate (white asterisk, cell body; white arrows, leading process). Cell is double-labeled for somatostatin (green) and A_{2A}R (red). (G and H) A_{2A}R immunolabeling of a representative coronal section of the monkey fetal telencephalon at E75. (G) A_{2A}R immunoreactivity was detected in the lower intermediate zone (IZ)/upper subventricular zone (arrowheads). (H) Higher magnification of the region in (G) showing many tangentially oriented migrating neuron-like cells immunolabeled for A_{2A}R (black arrows). Scale bars, 50 μ m (A to C), 5 μ m (D to F), 250 μ m (G), and 25 μ m (H).



0.1 ml of flurothyl was applied in the cage (Table 2). However, two pups from each of the treated group (caffeine and KW6002) displayed aberrant movements, whereas none of the control animals did. When 0.2 ml of flurothyl was applied in the cage, only 6 abnormal movements were observed in the control group, whereas all treated pups made a total of 43 abnormal movements. At this flurothyl concentration, 100% of treated pups displayed seizures versus 38% in the control group. The time to trigger seizures, as well as the duration of seizures, was similar in treated and nontreated offspring (Table 2). We conclude that animals exposed to caffeine or KW6002 during the developmental period are more susceptible to seizures than controls, in agreement with the hyperexcitability of hippocampal networks found in vitro.

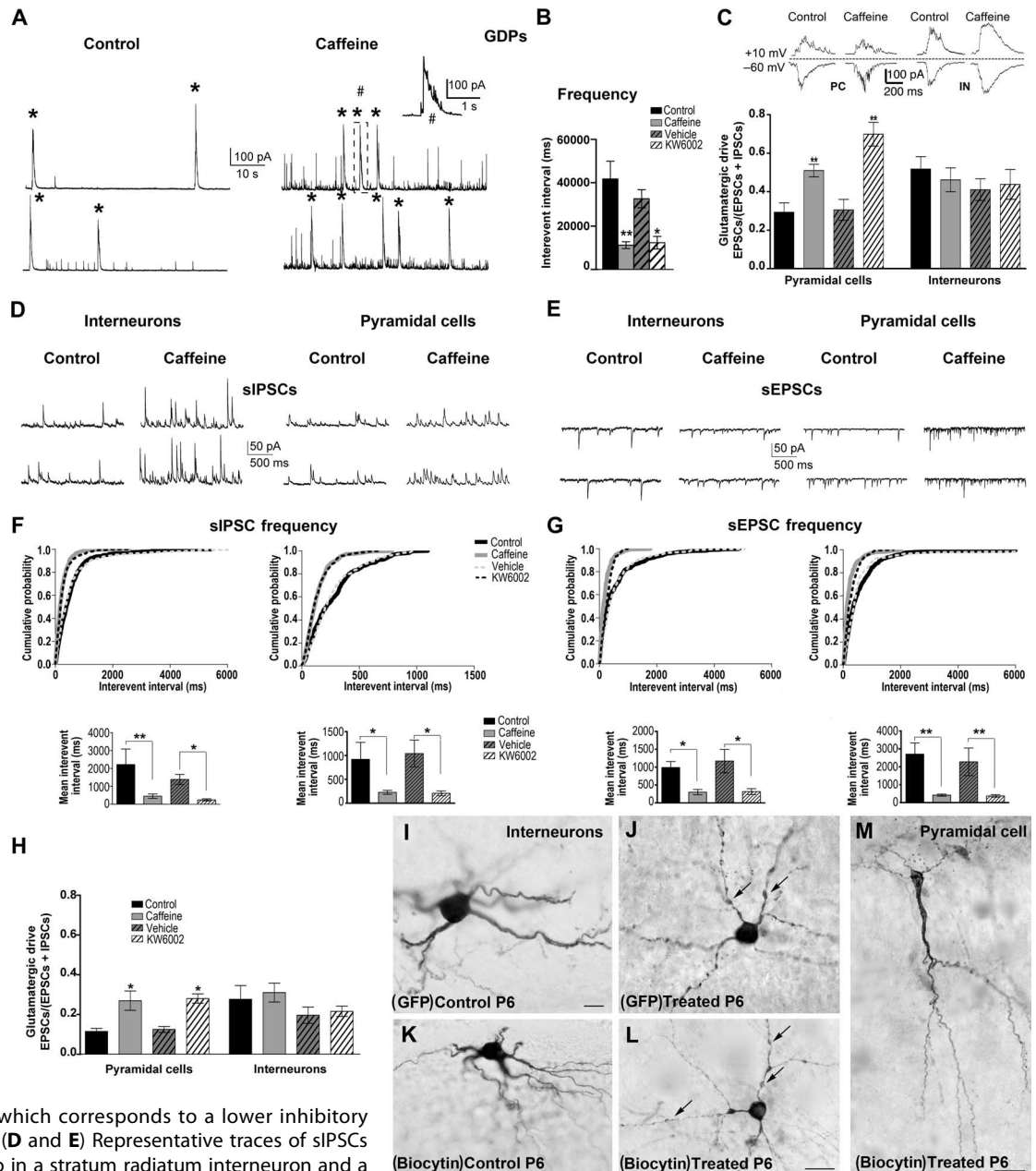
Interfering with GABAergic circuits during development alters neuronal networks in the adult at both structural and functional levels, and leads to cognitive deficits (31, 40, 41). We thus analyzed whether caffeine or KW6002 exposure led to long-term sequelae in adult hippocampal networks and assessed possible functional consequences.

Hippocampal neuronal networks are altered in adult rodent offspring

Using immunohistochemistry for the neuron-specific nuclear protein (NeuN), we found a marked neuronal loss in the stratum oriens and stratum radiatum/lacunosum moleculare of the CA1-CA3 region as well as in the hilus of the dentate gyrus of caffeine-exposed ($n = 7$)

Fig. 3. Caffeine exposure increases spontaneous and network glutamatergic and GABAergic activities in P6 mouse offspring.

(A) Representative traces of spontaneous activity recorded at a holding potential of +10 mV in a hippocampal CA3 PC from a brain slice of a control or a caffeine-exposed pup at P6. Black asterisks represent GDPs. Note the increase in the frequency of GDPs and of spontaneous synaptic currents recorded between GDPs in caffeine-exposed offspring. Inset: Detail of a GDP event indicated by #. **(B)** Bar graphs showing a decreased mean IEI between GDPs recorded in hippocampal brain slices from caffeine-exposed pups compared to control pups (** $P < 0.01$) and from KW6002-exposed pups compared to vehicle (* $P < 0.05$). **(C)** The glutamatergic contribution to the overall synaptic input during GDPs was increased in PCs (** $P < 0.01$) but not in the stratum radiatum (R) interneurons (IN) ($P = 0.82$) from caffeine- or KW6002-exposed pups. The top panel shows representative glutamatergic and GABAergic currents recorded at -60 and +10 mV, respectively, during GDPs in a CA3 PC and a stratum radiatum interneuron. The area under the curve was smaller in GDPs recorded at +10 mV in PCs of caffeine-exposed pups, which corresponds to a lower inhibitory charge transfer to the cells. **(D and E)** Representative traces of sIPSCs and sEPSCs recorded in vitro in a stratum radiatum interneuron and a PC from control and caffeine-exposed offspring. **(F and G)** Cumulative probability distributions of IEIs for sIPSCs and sEPSCs recorded in vitro in a stratum radiatum interneuron and a PC from control and caffeine-exposed mouse offspring. Bar graphs showing the mean IEI are displayed below the cumulative probability distributions. In hippocampal slices from caffeine- or KW6002-exposed offspring, the IEI of sIPSCs (left panels) and sEPSCs (right panels) was decreased in stratum radiatum interneurons and in PCs compared to control or vehicle-exposed animals. **(H)** The contribution of the glutamatergic synaptic input to the overall synaptic input was increased selectively in PCs from caffeine- and KW6002-exposed offspring, but this was not observed for stratum radiatum interneurons (PCs, * $P < 0.01$; interneurons, $P = 0.23$). **(I and J)** GFP-labeled neurons in a control (I) and in a caffeine-exposed (J) P6 pup (same as in Fig. 1). **(K to M)** Biocytin-labeled interneurons (I and J) and PCs (K) recorded in vitro in a control (K) and a caffeine-exposed (L and M) P6 pup. In neurons from caffeine-exposed offspring, varicosities were consistently observed along dendrites of interneurons labeled with GFP (J, black arrows) or with biocytin (L, black arrows) but not along dendrites of PCs (M). These varicosities on dendrites are reminiscent of swelling profiles observed after excitotoxic damage. Scale bars, 50 μm (I and J) and 25 μm (K to M).



and KW6002-exposed ($n = 4$) adult offspring (3 months old) compared to control ($n = 12$) mice (Fig. 4, A and B, and Table 3). The estimated total number of neurons in caffeine- or KW6002-exposed animals was

decreased, respectively, by 38 and 19% in the stratum oriens, by 34 and 24% in stratum radiatum/lacunosum moleculare, and by 39 and 22% in the hilus of the dentate gyrus (Fig. 4E and Table 3). Given that, in con-

Table 2. Seizure susceptibility of control and caffeine- or KW6002-exposed pups to the convulsing agent flurothyl. Caffeine- or KW6002-exposed P6 pups were more vulnerable to flurothyl than control pups of the same age (eight controls from two dams, four caffeine-exposed pups from one dam, and four KW6002-exposed pups from one dam). Treated pups displayed a higher number of abnormal (spasm-like) movements after exposure to flurothyl, and the proportion of treated pups that manifested clonic seizures at a lower concentration of flurothyl (0.2 ml) was greater. No differences were found between the time to trigger seizures and the duration of seizures between groups.

	0.1-ml flurothyl		0.2-ml flurothyl	
	Control	Caffeine/ KW6002	Control	Caffeine/ KW6002
No. abnormal movements/session	0	2	6	43
% Animals displaying seizures	0	0	37.5	100
Time to trigger seizures (min)	—	—	13.5 ± 0.5	13.4 ± 0.5
Duration of seizures (min)	—	—	1.8 ± 1.1	1.4 ± 0.2

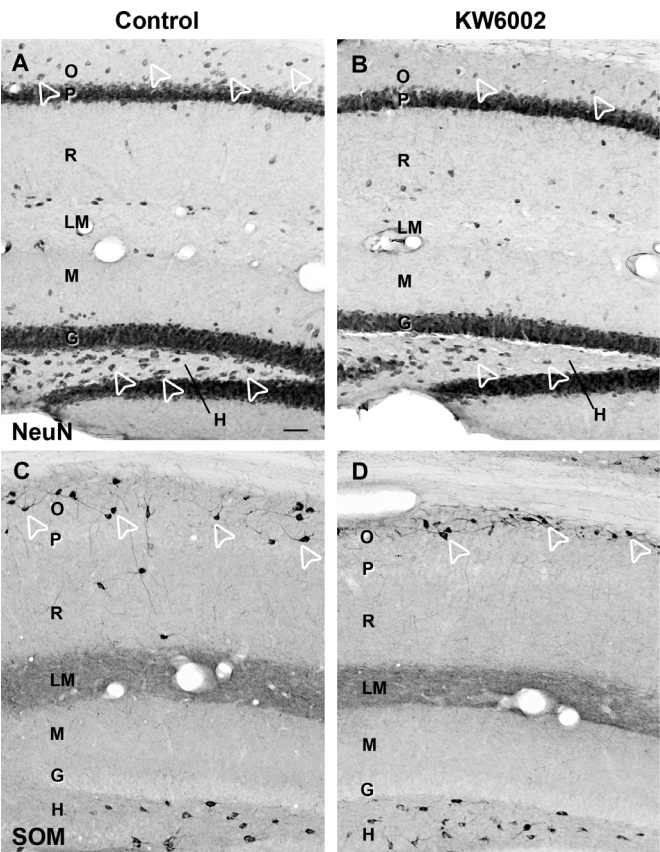
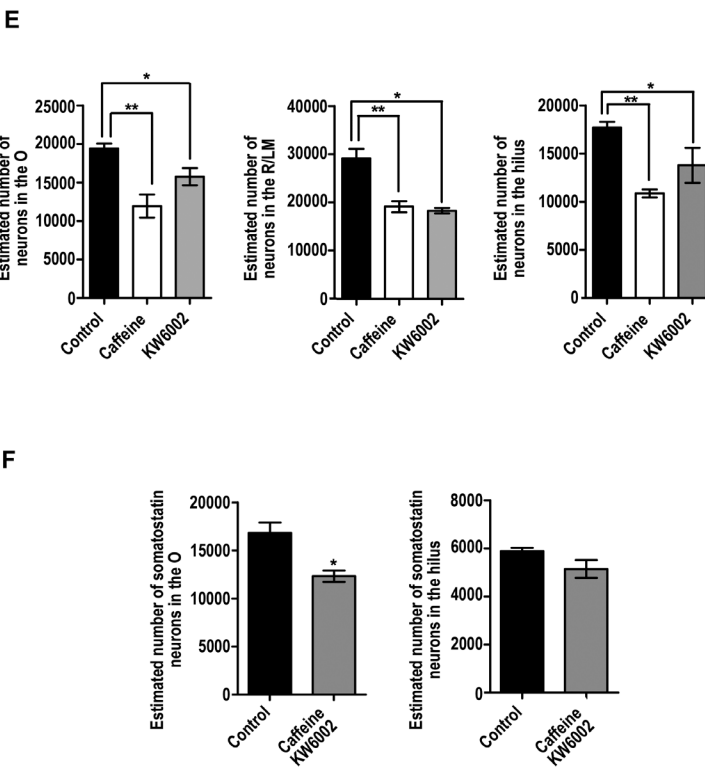


Fig. 4. Caffeine treatment leads to GABAergic cell loss in adult offspring. (A to D) Representative hippocampal coronal sections (of 10 to 12 examined per animal) from control (A and C) and KW6002-exposed (B and D) adult mouse offspring immunolabeled for NeuN (A and B) and somatostatin (C and D). (A and B) NeuN-labeled cell bodies (arrowheads) were decreased in the stratum oriens (O) and the hilus (H) of the dentate gyrus in KW6002-exposed offspring (B) compared to controls (A). (D) KW6002-exposed offspring showed a decrease in somatostatin-containing neurons (arrowheads) compared to control (C). (E) Bar graphs of the estimated number of NeuN-containing neurons in control and caffeine- or

control GIN mice, 87% of the stratum oriens neurons contain somatostatin, a population of neurons known to be particularly vulnerable in pathological conditions (42–45), we specifically analyzed their fate in adult caffeine-exposed offspring. Most of the cell loss observed in the stratum oriens corresponded to a 30% decrease in somatostatin-containing GABA neurons (Fig. 4, C, D, and F, and Table 3). The loss of somatostatin-containing neurons was region-specific because no difference in the number of somatostatin-containing neurons was found in the hilus of the dentate gyrus between treated (Fig. 4, D and F, and Table 3) and control mice (Fig. 4, C and F, and Table 3). The cell loss was also cell type-specific, because the number of GFP-containing interneurons, which represent a subpopulation of somatostatin-containing neurons in GIN mice, was not modified in adult animals (Fig. 1).

We then investigated whether the loss of somatostatin-containing interneurons in the stratum oriens was associated with electrophysiological modifications in CA1 PCs, the main target of these neurons and the output region of the hippocampal network. The frequency of sIPSCs was increased by 155 and 252% in caffeine- and KW6002-exposed adult offspring, respectively (Fig. 5, A and C, and table S5), whereas the



KW6002-exposed offspring show a significant decrease in the number of neurons in caffeine- and KW6002-exposed adult offspring compared to control in the stratum oriens (caffeine, $^{***}P < 0.01$; KW6002, $^{*}P < 0.05$), radiatum/lacunosum moleculare (R/LM) (caffeine, $^{***}P < 0.01$; KW6002, $^{*}P < 0.05$), and hilus (caffeine, $^{***}P < 0.01$; KW6002, $^{*}P < 0.05$). (F) Bar graphs of the estimated number of somatostatin-containing neurons show a significant decrease in the number of neurons in caffeine- and KW6002-exposed offspring compared to control in the stratum oriens ($^{*}P < 0.05$) but not in the hilus ($P = 0.132$). P, stratum pyramidale; M, molecular layer of the dentate gyrus; G, granular cell layer. $^{*}P < 0.05$, $^{***}P < 0.01$. Scale bars, 50 μ m (A to D).

Table 3. Loss of neurons in treated adult offspring. Stereological quantification of the total number of neurons (identified by NeuN immunoreactivity) and somatostatin-expressing neurons in the hippocampus is shown for control, caffeine-exposed, and KW6002-exposed adult mouse offspring. NeuN-labeled neurons were quantified in the hippocampal regions (stratum oriens,

hilus of the dentate gyrus, and stratum radiatum). Somatostatin-expressing interneurons were quantified in the hilus of the dentate gyrus and stratum oriens, where they were more abundantly located. Given that vehicle treatment did not modify any morphological parameter measured at P6, a stereological quantification was not performed in this group of adult animals.

	Stratum oriens			Dentate gyrus (hilus)			Stratum radiatum		
NeuN	Control	Caffeine	KW6002	Control	Caffeine	KW6002	Control	Caffeine	KW6002
	19,404 ± 658 (n = 4)	11,938 ± 1,506 (n = 4) [†]	15,744 ± 1,113 (n = 3)*	17,670 ± 629 (n = 4)	10,866 ± 411 (n = 4) [†]	13,776 ± 1,815 (n = 3)*	29,154 ± 1,988 (n = 4)	19,152 ± 1,152 (n = 4) [†]	22,064 ± 3,778 (n = 4)*
Somatostatin	Control	Caffeine/KW6002		Control	Caffeine/KW6002				
	16,817 ± 1,072 (n = 3)	12,296 ± 592 (n = 3) *		5,884 ± 140 (n = 3)	5,141 ± 368 (n = 3)				

*P < 0.05, one-way ANOVA. †P < 0.01, one-way ANOVA.

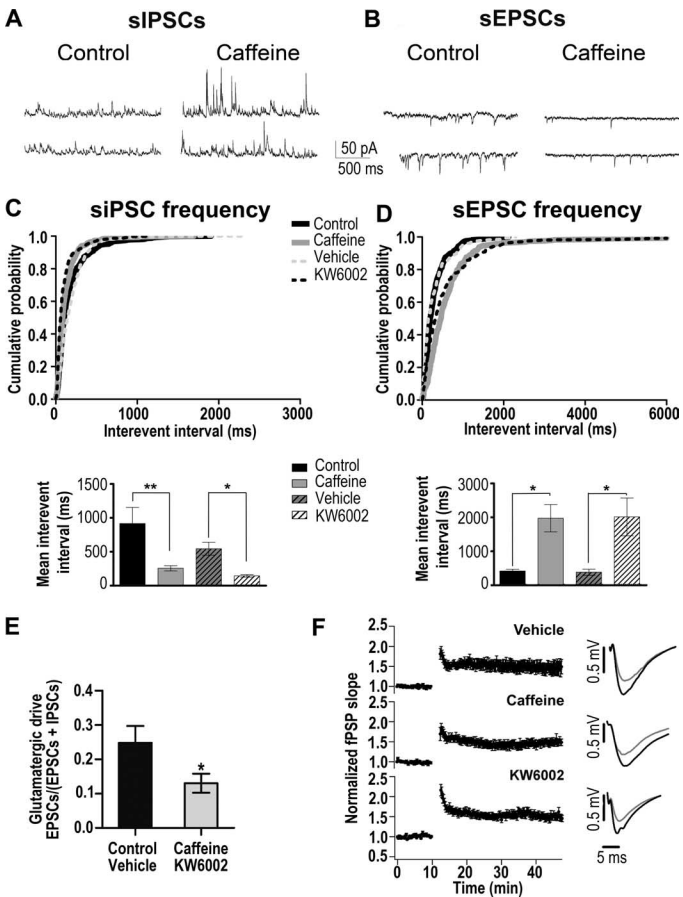


Fig. 5. GABAergic and glutamatergic activity in hippocampal CA1 PCs in adult offspring. (A) The frequency of sIPSCs was increased in caffeine-exposed adult offspring representing an increase in GABAergic activity. (B) The frequency of sEPSCs was decreased in caffeine-exposed adult offspring representing a decrease in glutamatergic activity. (C and D) Cumulative probability distributions of IELs for sIPSCs and sEPSCs in caffeine- and KW6002-exposed adult offspring. Histograms showing the mean IELs are depicted below. (E) The glutamatergic synaptic input was decreased in PCs of caffeine- and KW6002-exposed animals (*P < 0.05). (F) LTP is similar in control and caffeine- and KW6002-exposed adult mouse offspring. Left: Time evolution of field postsynaptic potential (fPSP) slopes after tetanic stimulation applied 10 min after obtaining stable baseline. Right: Averaged fPSP slopes before (gray trace) and 30 min after (black trace) tetanic stimulation. *P < 0.05, **P < 0.01.

frequency of sEPSCs was decreased by 79 and 82% in adult caffeine- and KW6002-exposed adult offspring, respectively (Fig. 5, B and D, and table S5). The corresponding mean decrease in the IEL between consecutive synaptic events is displayed by the histograms below the frequency curves. As a result, the contribution of the glutamatergic synaptic input to the overall synaptic input was decreased by 48% in caffeine- and KW6002-exposed compared to control and vehicle-treated animals (Fig. 5E). This may reflect a reorganization of the hippocampal circuitry in adult caffeine-exposed offspring, which may involve a hypoactivity of glutamatergic networks as well as a hyperactivity of GABAergic networks (33, 34, 42). These modifications were not associated with changes in long-term potentiation (LTP). LTP was induced in 6 of 9 hippocampal slices in controls, in 8 of 12 slices in caffeine-exposed, and in 8 of 8 slices in KW6002-exposed adult offspring (Fig. 5F). The increase in the slope of the field excitatory postsynaptic potential (that is, LTP) was not significantly different (P = 0.74) between controls (47%), caffeine-exposed (37%), and KW6002-exposed (51%) adult offspring (Fig. 5F). The stimulus intensity used to evoke field potentials was similar in the three groups, suggesting that the decrease in sEPSC frequency (Fig. 5, B and D) reflects a hypoactivity of presynaptic glutamatergic networks rather than their loss.

Caffeine-exposed rodent offspring display cognitive deficits as adults

Given that the reorganization of hippocampal circuits may be associated with deficits in spatial memory (46), we assessed hippocampus-dependent memory in treated and control adult offspring using an object displacement and substitution task. Animals exposed to caffeine during development showed a lower preference for the displaced object compared to control animals, indicating a deficit in spatial, hippocampus-dependent memory (P < 0.05, nonparametric t test) (Fig. 6A). Their performance was also decreased on the nonspatial memory task, as they spent similar times exploring the familiar and new object (P < 0.05, nonparametric t test) (Fig. 6B). Locomotor activity was not altered in caffeine-exposed adult offspring (Fig. 6C). However, caffeine-exposed adult offspring displayed impairment in the novel object recognition test (Fig. 6D) and two-trial reference memory test in the Y maze (Fig. 6E), in keeping with the results obtained in rats exposed to caffeine during the embryonic period only (47). The recognition index for the novel object was decreased by 40% in caffeine-exposed and by 64% in KW6002-exposed adult offspring (Fig. 6D; P < 0.05 control versus caffeine, and P < 0.01 vehicle

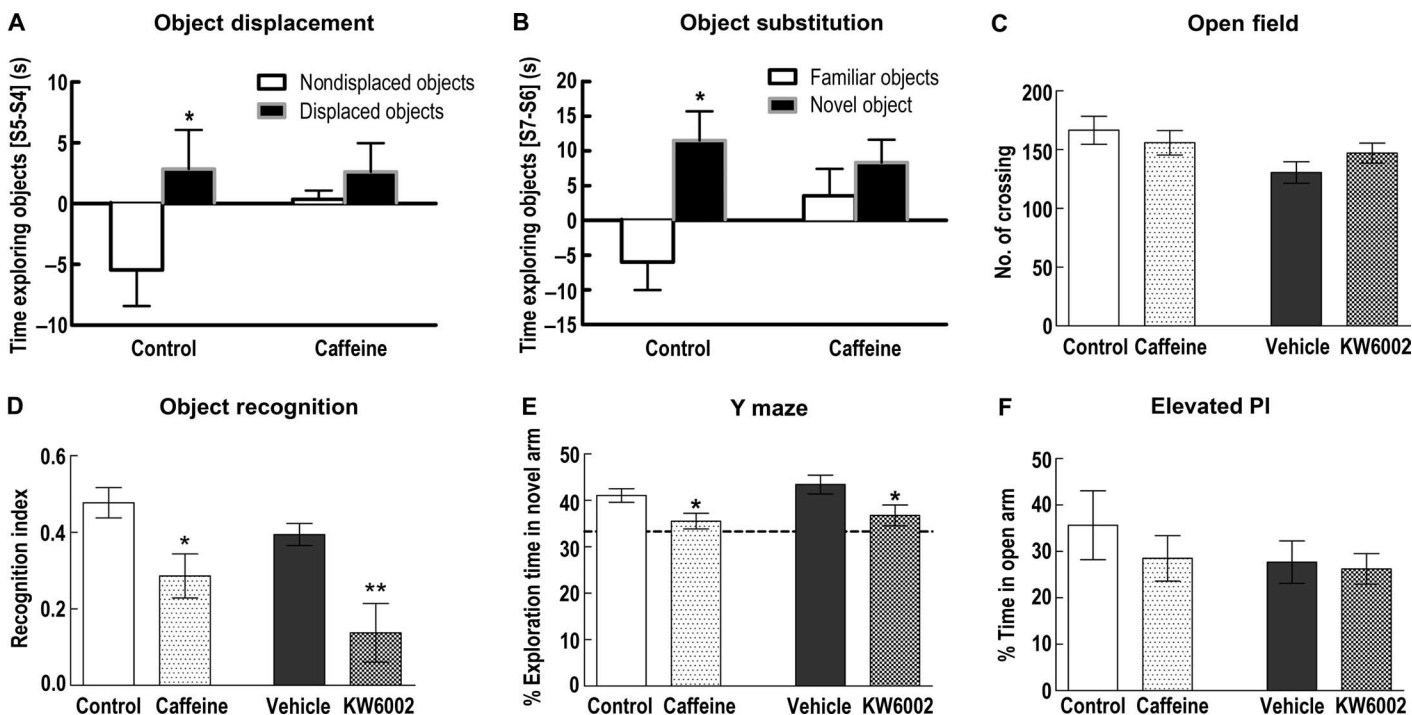


Fig. 6. Caffeine exposure during early life leads to memory deficits in adult offspring. (A) Caffeine-exposed adult offspring (6 months of age, $n = 5$) displayed a deficit in hippocampus-dependent spatial memory on the object displacement task compared to control ($n = 7$) (A, control, nondisplaced: -5.46 ± 2.96 , displaced: 2.85 ± 3.22 , $*P < 0.05$; caffeine, nondisplaced: 0.36 ± 0.72 , displaced: 2.61 ± 2.36 , $P = 0.286$). (B) Caffeine-exposed adult offspring (6 months of age) also displayed a deficit on the hippocampal-dependent nonspatial object substitution task compared to control (control, $n = 7$, familiar: -6.02 ± 4.01 , novel: 11.51 ± 4.20 , $*P < 0.05$; caffeine, $n = 5$, familiar: 3.55 ± 3.87 , novel: 8.32 ± 3.31 , $P = 0.090$). (C to E) Behavior analysis of control, caffeine-exposed, vehicle-exposed, and KW6002-exposed adult GIN mouse offspring (at 3 months of age). (C) Locomotion (number of crossings) of animals exposed to caffeine or KW6002, analyzed during the first session in the open-field arena, was similar to respective controls (control:

166 ± 12 , $n = 9$; caffeine: 156 ± 10 , $n = 8$, $P = 0.588$; vehicle: 131 ± 9 , $n = 10$; KW6002: 147 ± 8 , $n = 7$, $P = 0.227$). (D and E) In contrast to their respective controls, both caffeine- and KW6002-exposed adult offspring displayed impaired performance on memory tasks in the object recognition test (D, control: 0.48 ± 0.04 , $n = 9$, caffeine: 0.29 ± 0.06 , $n = 8$, $*P < 0.05$, vehicle: 0.39 ± 0.003 , $n = 10$, KW6002: 0.14 ± 0.08 , $n = 7$, $**P < 0.01$) and by spending less time exploring the novel arm in the Y maze (E, control: $41 \pm 1\%$, $n = 8$, caffeine: $36 \pm 2\%$, $n = 10$, $*P < 0.05$; vehicle: $43 \pm 2\%$, $n = 10$, KW6002: $37 \pm 2\%$, $n = 7$, $*P < 0.05$). (F) All treated and untreated adult offspring spent a similar amount of time in the open arm of the elevated plus maze, indicating no change in anxiety (control: $37 \pm 7\%$, $n = 9$, caffeine: $29 \pm 5\%$, $n = 10$, $P = 0.423$; vehicle: $28 \pm 5\%$, $n = 10$, KW6002: $26 \pm 3\%$, $n = 7$, $P = 0.817$). $*P < 0.05$, $**P < 0.01$, one-way ANOVA followed by a Tukey test, comparing treated with control mice.

versus KW6002). In the modified Y-maze test, all treated animals spent less time exploring the novel arm during the test trial. The exploration time decreased from 41 to 36% in caffeine-exposed and from 43 to 37% in KW6002-exposed adult offspring (Fig. 6E; $P < 0.05$). Altered performances in these tasks were not related to increased anxiety because there was no difference between control, vehicle-treated, caffeine-treated, and KW6002-treated mice in the time spent in the open arm in the elevated plus maze (Fig. 6F). These data demonstrate that early life exposure to caffeine (or KW6002) may lead to altered memory function.

DISCUSSION

The results presented here suggest that maternal caffeine consumption by mouse dams during gestation and lactation has effects on neuronal development and adult behavior of their offspring. This developmental effect of caffeine may involve the blockade of $A_{2A}R$ s because caffeine-induced modifications could be reproduced with a selective $A_{2A}R$ antagonist, although we cannot rule out contributions of the other targets

of caffeine, for example, A_1 receptors, as shown during parturition (48). Caffeine treatment did not have a major influence on food and water intake or on the day/night rhythm of female mice. However, we cannot fully exclude that treatment-associated physiological or behavioral changes in dams might have influenced fetal and/or neonatal development, for example, through changes in placental perfusion (49). During the dark, that is, active phase, female caffeine-treated mice showed increased activity. However, dams and pups were always found in the nest, suggesting that increased maternal activity did not impair maternal care for pups. The fact that we were able to reproduce our data in $A_{2A}R$ -KO pups suggests that the impact of perinatal caffeine exposure on brain development may be linked to antagonism of $A_{2A}R$ by caffeine. It has been reported that $A_{2A}R$ -KO adult mice display enhanced working memory (50) but not reference memory (51). This finding does not contradict our results as we stopped the transient interference of $A_{2A}R$ s after weaning by withdrawing caffeine from the drinking water, whereas $A_{2A}R$ s are absent throughout life in the $A_{2A}R$ -KO animals. Thus, our results suggest a new role for $A_{2A}R$ s in the control of hippocampal development. However, we cannot fully rule out that the

caffeine-induced maternal hyperactivity and weight loss of the pups might be a potentially confounding factor. In keeping with our observations, maternal caffeine intake during pregnancy in humans is associated with lower birth weight but no change in gestational length (52). In humans, lower birth weight correlates with enhanced seizure susceptibility (53) and cognitive deficits (54).

The delayed migration of GABA neurons induced by caffeine exposure was associated with a general increase in neuronal network activity, although causality between both observations remains to be established. These alterations share some similarities with those found in animals exposed to drugs of abuse during developmental periods (6). After weaning, animals were not exposed to caffeine any longer, but the early effects of caffeine on neuron migration and integration ultimately led to long-term sequelae in hippocampal circuits. The disruption of developmental programs usually leads to long-term alterations in GABAergic circuits, typified by neuronal loss (including subpopulations of somatostatin-containing neurons), and cognitive deficits in adult animals (40, 55–57). The phenotypic traits observed after caffeine exposure show some similarities to those found in models of neuronal migration disorders (31) and models of psychiatric disorders linked to GABA neuron dysfunction (41). This suggests that interfering with GABA neurons during development may have adverse consequences.

The mechanisms underlying the changes in glutamatergic and GABAergic activities in adult caffeine-exposed offspring may involve an imbalance between excitation and inhibition; a correct balance is important for the proper functioning of cortical networks (58, 59). Cognitive deficits in certain psychiatric disorders are also associated with hyperactivity of inhibitory circuits or hypoactivity of glutamatergic circuits (41). The altered balance between excitation and inhibition found in adult caffeine-exposed offspring may contribute to the impaired memory observed on several memory tests.

The challenge with rodent models for studying the effects of psychoactive drugs is whether the results can be extrapolated to humans given the differences in development and maturation across species. The expression of A_{2A}Rs in migrating neurons in macaques indicates that A_{2A}R-related pathways can be potentially affected by caffeine in primates. Note that caffeine citrate is widely used for the treatment of apnea in premature human neonates up to 33 to 34 weeks of gestation. No deleterious consequences of this treatment have been identified in treated premature neonates up to the age of 5 years (60, 61). Given our findings, it will be important to develop longitudinal clinical studies to assess short- and long-term consequences in human neonates born to women who consumed caffeine on a regular basis during pregnancy and lactation, as well as in neonates treated with caffeine citrate.

MATERIALS AND METHODS

Study design

The overall objective of the study was to determine the effects of caffeine consumption (caffeine was added to the drinking water) by mouse dams on the brain development of their offspring. Hippocampal circuits of male offspring at P6 from treated and untreated dams were assessed with electrophysiological, morphological, and immunohistochemical techniques. Seizure susceptibility was also assessed. When the caffeine-exposed and control offspring reached adulthood, we assessed hippocampal circuits and cognitive performance. A specific antagonist of A_{2A}Rs was also used to assess whether the effects of caffeine could be

due to its antagonizing action on A_{2A}Rs. Time-lapse imaging was used to measure the effects of caffeine and A_{2A}R antagonists on the migration of GABA neurons in medial ganglionic eminence cell cocultures. Finally, several cognitive functions, including spatial memory, were assessed in caffeine-exposed adult offspring.

Animals

Caffeine and KW6002 treatments were performed in GIN mice and in A_{2A}R constitutive knockout dams. Caffeine was given to animals dissolved in tap water at a concentration of 0.3 g/liter. KW6002 (istradefylline) was prepared and given to animals at a concentration of 2 mg/kg per day in a vehicle solution containing 0.4% methylcellulose and 0.9% NaCl. Both treatments were started 15 days before mating and prolonged during all gestational period up to P15. Caffeine treatment started at gestational day 0.5 for experiments assessing the activity of females and behavioral analysis of pups of treated dams.

Electrophysiology

Transverse hippocampal slices from pups and from adults. IPSCs were recorded at a holding potential of +10 mV, the reversal potential for glutamatergic events, and EPSCs were recorded at –60 mV, the reversal potential for GABAergic events (42). For LTP induction protocol, fPSPs were recorded in the stratum radiatum of CA1 region and evoked upon tetanic stimulation of the Schaffer collaterals.

Immunohistochemistry

Immunohistochemistry was performed as previously described (30, 62) with tissue from rat embryos, GIN mouse pups, A_{2A}R-KO pups, and adult GIN mice. Immunohistochemical localization of A_{2A}Rs was performed in E13 Wistar rats and E75 cynomolgus monkey (*Macaca fascicularis*).

Stereological quantification

The numbers of neurons labeled for NeuN, GFP, and somatostatin were estimated with the optical fractionator method, as previously described (43).

Cell culture of MGE and analysis of migration speed

GFP-expressing MGE explants (E13.5) were cultured on E13.5-dissociated cortical cells (26) and monitored by time-lapse video microscopy before and after drug application.

Measurement of caffeine and its metabolites in brain tissue

The tissue of all brain was homogenized and mixed with 30% of perchloric acid, and the supernatant obtained after centrifugation was separated by high-performance liquid chromatography (HPLC).

Behavioral analysis

Locomotor activity was evaluated in an open-field arena by counting the total number of crossings for 5 min. Spatial memory performance was evaluated with the modified Y-maze test (63, 64). The object recognition test was performed, and the recognition index was calculated as previously described (65, 66). Anxiety was evaluated in an elevated plus maze (67, 68). The object displacement and substitution task tests were performed in three stages (69).

Seizure induction with flurothyl

The evaluation of spasm-like movements and seizures was based on (70). Animals were video-monitored.

Statistical analysis

One-way ANOVA was used to compare the number of neurons and the physiological activity in the hippocampus of control, vehicle-exposed, caffeine-exposed, and KW6002-exposed pups and adults. Eventually, a Tukey test was used as a post hoc test. A Student's *t* test was used in the experiments of cell migration and in the object displacement and substitution task test. The behavioral parameters of female mice and pups were analyzed with a three-way or two-way mixed ANOVA, respectively, followed by Newman-Keuls post hoc tests when appropriate. Statistical significance was considered when $P < 0.05$. Levels of significance are indicated as follows: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

SUPPLEMENTARY MATERIALS

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Methods

Fig. S1. Physiological and behavioral consequences of caffeine treatment.

Fig. S2. Caffeine exposure during pregnancy and the early postnatal period delays the migration and insertion of GFP-containing neurons in cortical layers and in the hippocampus.

Fig. S3. Caffeine exposure during pregnancy and the early postnatal period delays the migration and insertion of somatostatin-containing neurons and the maturation of the hippocampal GABAergic network.

Fig. S4. Decreased numbers of somatostatin-containing neurons in the hippocampus and in cortical layers of mice lacking A_{2A} Rs.

Table S1. Properties of the spontaneous activity (GDPs) in hippocampal slices from P5 to P7 control, caffeine-exposed, vehicle-exposed, and KW6002-exposed mouse pup offspring.

Table S2. Imbalance between excitatory and inhibitory synaptic inputs in PCs from caffeine- and KW6002-exposed pup offspring during GDPs.

Table S3. Properties of the spontaneous activity recorded in vitro in hippocampal neurons of P5 to P7 mouse pup offspring.

Table S4. Imbalance between excitatory and inhibitory synaptic inputs in PCs from caffeine- and KW6002-exposed pup offspring during spontaneous activity outside GDPs.

Table S5. Properties of the spontaneous activity recorded in vitro in hippocampal neurons from control, caffeine-exposed, vehicle-exposed, and KW6002-exposed adult mouse offspring.

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