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Caffeine alters proliferation of neuronal precursors in the adult hippocampus

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Abstract

Neurogenesis continues through adulthood in the hippocampus and olfactory bulb of mammals. Adult neurogenesis has been implicated in learning and memory, and linked with depression. Hippocampal neurogenesis is increased in response to a number of stimuli, including exposure to an enriched environment, increased locomotor activity, and administration of antidepressants. Adult neurogenesis is depressed in response to aging, stress and sleep deprivation. Intriguingly, caffeine modulates a number of these same stimuli in a dose-dependent manner. We examined the dose and duration dependent effects of caffeine on the proliferation, differentiation, and survival of newly generated hippocampal neurons in adult mice. Extended, 7-day caffeine administration, alters the proliferation of adult hippocampal precursors in the mouse in a dose dependent manner; moderate to high doses (20–30mg/kg/day) of caffeine depress proliferation while supraphysiological doses (60mg/kg/day) increase proliferation of neuronal precursors. Acute, 1-day administration had no affect on proliferation. Caffeine administration does not affect the expression of early or late markers of neuronal differentiation, or rates of long-term survival. However, neurons induced in response to supraphysiological levels of caffeine have a lower survival rate than control cells; increased proliferation does not yield an increase in long-term neurogenesis. These results demonstrate that physiologically relevant doses of caffeine can significantly depress adult hippocampal neurogenesis.

Keywords

Adult Neurogenesis; Caffeine; Hippocampus; cleaved caspase-3; differentiation

1. Introduction

One of the mechanisms underlying the adult brain's plasticity is neurogenesis, the addition of new neurons to already existing neural circuits. Neurogenesis continues through adulthood in the olfactory bulb (Altman, 1969; Lois and Alvarez-Buylla, 1994) and dentate gyrus of the hippocampus (Altman and Das, 1965) in mammals, including humans (Eriksson et al., 1998). The plasticity provided by newly generated neurons likely mediates aspects of learning and memory (Magavi et al., 2005; Shors et al., 2001) and the relationship between neurogenesis and depression is being extensively explored (Jacobs et al., 2000). Learning tasks, enriched environment (Kempermann et al., 1997), running, and antidepressants (Malberg et al., 2000) increase neurogenesis while aging (Cameron and McKay, 1999; Kuhn et al., 1996), stress

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(Gould et al., 1997), and sleep deprivation (Guzman-Marín et al., 2005; Mirescu et al., 2006) depress hippocampal neurogenesis. Behavioral and pharmacological factors modulating adult neurogenesis may, in turn, have important influences on learning and depression.

Caffeine, one of the most common psychoactive substances ingested by humans, has complex behavioral and biochemical effects in the brain and throughout the body (Fredholm et al., 1999) that could influence neurogenesis. As a stimulant, caffeine often increases alertness and can improve performance on tasks requiring sustained attention (Smith, 2002). Administration of caffeine increases locomotor activity, with a maximal increase at about 30 mg/kg in rodents and a reduction in activity at higher doses (Finn and Holtzman, 1986). Caffeine has also been shown to exert neuroprotective effects in models of Parkinson's disease (Chen et al., 2001; Ross et al., 2000). Higher doses of caffeine can have negative effects: they can induce difficulty in falling asleep and disturbances in sleep, are associated with increased anxiety in humans (Chait, 1992) and rodents, and can induce panic attacks in susceptible patients (Clementz and Dailey, 1988).

Caffeine affects behaviors and biochemical pathways that have been shown to influence the rate of neurogenesis in the adult hippocampus. At moderate doses, caffeine increases locomotor activity (Fredholm et al., 1999), which has been shown to increase neurogenesis in the dentate gyrus (van Praag et al., 1999). Caffeine can also contribute to sleep deprivation and anxiety or stress at higher doses, both of which have been associated with depressed adult neurogenesis (Guzman-Marín et al., 2005; Tanapat et al., 2001). Less directly, caffeine's vasoconstrictive effects could alter the vascular neurogenic niche in the brain (Palmer et al., 2000) and modify adult neurogenesis. Recent results from Han et al. (Han et al., 2007) showed that extended administration of a single dose of caffeine depressed neurogenesis in the adult rat hippocampus. We set out to systematically test the effects of caffeine on the proliferation, differentiation, and survival of adult-born hippocampal neurons over a broad range of doses.

We examined the effects of acute and extended caffeine administration on hippocampal neurogenesis. Caffeine's effects on proliferation and survival of adult-generated hippocampal neurons were examined using the thymidine analogue BrdU to label newly born cells. Differentiation was assessed using doublecortin (Dcx), a protein preferentially expressed by immature neurons (Gleeson et al., 1999), and NeuN, which is exclusively expressed by mature neurons. Originally, we hypothesized that physiologically relevant doses of caffeine, which generally increase activity, would upregulate neurogenesis while supraphysiological doses would depress neurogenesis. In fact, our results demonstrate the opposite – physiologically plausible doses of caffeine depressed neurogenesis while supraphysiological doses increased it.

2. Materials and Methods

2.1. Animals

All experiments were performed in accordance with the National Institutes of Health Guide and Use of Laboratory Animals, and approved by the Massachusetts Institute of Technology Committee on Animal Care. Eight-week-old female C57/BL6 mice (Charles River Labs) were used for all experiments. Three to five mice were housed per cage under standard conditions, on a twelve hour light-dark cycle at 23° C, and were provided food and water ad libitum. Control mice were maintained in parallel with experimentals to reduce variability.

2.2. Caffeine Treatments

For each dosage examined, caffeine (Sigma) was diluted in sterile saline such that the final volume each mouse received was 0.02 ml per gram of body weight. Thus, the average mouse received 0.4 ml of caffeine solution in each of the experimental groups.

Group I: Proliferation—In the extended exposure experiments, mice were injected intraperitoneally with a total of 10, 20, 25, 30 or 60 mg/kg of caffeine (Sigma) daily via two separate injections spaced 12 hours apart (each injection 5, 10, 12.5, 15 or 30 mg/kg, respectively) for 7 days. On the 8th day of the extended proliferation experiments, the mice received a 50 mg/kg injection of BrdU with their final dose of caffeine, a second injection of BrdU two hours later and were sacrificed at 4 hours. The mice received a total of 100 mg/kg of BrdU. Injections were staggered to ensure that mice were sacrificed precisely 4 hours after injection (Figure 1a). Sacrificing large numbers of mice on a tightly defined time course proved technically challenging, so we conducted multiple experiments, each with its own control to control for inter-experiment variability. A one-way ANOVA of all the control groups yielded a P-value of 0.8824, showing that there were no significant differences between the controls in our experiments.

In the acute exposure experiments, mice received a total of 60 mg/kg of caffeine via two 30 mg/kg doses separated by 12 hours (Figure 1b). The mice received an injection of BrdU with the second dose of caffeine (at time T), a second injection of BrdU two hours later (at T + 2 hours) and were sacrificed two hours following this last injection (at T + 4 hours).

Group II: Extended Caffeine – Early Differentiation—Mice were administered 60 mg/kg/day of caffeine for 7 days. On the 8th day, a single caffeine injection was given in the morning followed by 50 mg/kg BrdU injection. Progenitors were allowed to differentiate with continued caffeine treatment for three days (Figure 1c). We then assessed the percentage of newly generated, BrdU+ cells that expressed doublecortin, a protein expressed predominantly by immature neurons.

Group III: Extended Caffeine – Survival—To assess the effects of extended caffeine administration on the survival of adult-born hippocampal neurons, we first administered 50 mg/kg BrdU, then treated the mice with 10 or 30 mg/kg of caffeine twice daily for 28 days, and sacrificed them (Figure 1d). We quantified the percentage of BrdU+ positive cells expressing NeuN, a mature neuronal marker.

Group IV: Long-Term Differentiation and Survival—Of particular interest was assessing whether the newborn cells induced by extremely high dose caffeine survived, differentiated into neurons and yielded a genuine increase in neurogenesis. To this end, we treated mice with 30 mg/kg of caffeine twice daily for 10 days, administered BrdU twice daily on the last three days, and maintained the mice for 28 or 56 days without further caffeine treatment (Figure 1e).

2.3. Immunohistochemistry

Mice were sacrificed following a lethal dosage of Avertin. Animals were perfused with PBS followed by 3% paraformaldehyde. Brains were postfixed in paraformaldehyde overnight at 4°C and sectioned at 50 microns using a Leica VT1000 vibrating microtome. Experimental and control sections were stained in parallel. The sections were immunohistochemically stained with antibodies against BrdU (Accurate OBT0030G, 1:500), Doublecortin (Santa Cruz sc-8066: 1:800) and NeuN (Chemicon MAB377 1:400). To expose the BrdU antigen, we washed sections in 2M HCl for 2 hrs at room temperature. We used Molecular Probes' highly cross-adsorbed Alexa 488 chicken anti-rat, Alexa 546 donkey anti-goat, and Alexa 546 goat

anti-mouse secondary antibodies. Primary and secondary antibodies were diluted in a blocking solution containing 0.3% triton, 8% Goat serum and 0.3% BSA diluted in PBS. Figures were assembled using Adobe Photoshop and Illustrator.

2.4. Quantification

Quantification of cells was performed using an Olympus IX70 microscope and Q-imaging Retiga 1300i camera. Two independent observers blinded to the experimental status of the tissue counted the number of cells in every sixth 50-micron thick section through the rostro-caudal extent of the hippocampus, for a total of 11 sections. The entire dorsal and ventral blades of the dentate gyrus along the x-y axis were counted in each section, reducing artifacts due to subsampling of the region and increasing the volume sampled. A modified version of the stereological optical dissector technique was used; cells intersecting the upper boundary were excluded from the counts, minimizing over-counting. The top and bottom three microns of each section were excluded from analysis to reduce sectioning and vibratome chatter artifacts, yielding an optical dissector height of 44 microns. Partially or ambiguously stained cells were omitted from the analysis.

Double labeling was analyzed by a blinded observer using an Olympus Fluoview confocal microscope at 60x magnification. Every 12th section was examined and a minimum of 50 cells was examined from each of five mice for each condition. In our experience, Dcx immunohistochemistry exhibits a reduced intensity towards the center of 50 micron sections, likely due to limited antibody penetration. Thus, we analyzed the Dcx expression of BrdU positive cells in the outermost 20 microns of sections. To reduce bias, we attempted to assess the Dcx expression of every BrdU positive cell in the optically accessible regions each section.

2.5. Statistical Analyses

Experimental groups were compared to their simultaneously conducted controls via a non-paired two tailed Student's T-test. Since the control groups for each of the doses of caffeine did not significantly differ from each other, we pooled the control data for all the groups and examined the data using a one-way ANOVA followed by a Newman-Keuls post test. The t-test and the ANOVA were in agreement, showing statistical differences at the same doses.

Statistical comparisons in figure 2D and E, were performed using ANOVA tests and comparisons in figure 2B, C, F, and G were performed using a non-paired two-tailed Student's T-test. Data is presented as average $\pm$ SEM. * indicates $P < 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$.

The coefficient of error (C.E.), an estimate of the precision of stereological analysis, was computed using the Gundersen Jensen quadratic approximation.

3. Results

3.1 Caffeine influences proliferation in the dentate gyrus

Contrary to our expectations, we found that moderate to high doses of caffeine depress proliferation while only supraphysiological doses increase proliferation. A low dose of caffeine, 10 mg/kg/day, had no effect on proliferation. Moderate doses, between 20 and 25 mg/kg/day, depressed proliferation in the dentate gyrus by 20 to 25% (Figure 2a, Table 1). High doses of caffeine, 60 mg/kg/day, increased proliferation by over 50%. The results from two separately conducted experiments were pooled for the 60 mg/kg/day dosage. To explore the hypothesis that 60 mg/kg/day doses of caffeine could be inducing cell death in the hippocampus, we performed pilot experiments examining the number of cleaved-caspase 3 positive cells in the hippocampus. Using nonstereological techniques, we examined every sixth

section and directly visualized between 0 and 4 cleaved caspase-3-positive cells per series, which translates to roughly zero to twenty apoptotic cells per mouse. There was no significant difference between experimental and control mice. The large numbers of mice that would be required to demonstrate statistically reliable differences between the control and experimental groups via stereological analysis precluded us from pursuing these experiments further. However, these results suggest that the level of cleaved caspase-3 mediated apoptosis occurring at the timepoint we examined is relatively low.

To explore the duration of caffeine administration necessary to modify hippocampal proliferation, we also assessed proliferation after acute caffeine treatment. We gave two doses of caffeine, separated by 12 hours, and then assessed proliferation in the dentate gyrus (Figure 1b). A 60 mg/kg/day acutely administered dose of caffeine yielded no significant change in proliferation in the dentate gyrus (Figure 2b).

In addition to influencing proliferation, extended caffeine administration could potentially alter the differentiation of newly generated cells. To assess whether caffeine-induced cells differentiated into neurons at normal rates, we administered 60 mg/kg of caffeine per day for eight days, injected mice with BrdU, and allowed the progenitors to differentiate with continued caffeine treatment for three days (Figure 1c). We then assessed the percentage of newly generated, BrdU+ cells that expressed doublecortin, a protein preferentially expressed by immature neurons. In both control and experimental mice approximately 85% of BrdU+ cells differentiated into neurons as demonstrated by doublecortin staining (Figure 2c, 3a–c). Gross visual inspection of Dcx staining did not reveal any differences in morphology between neurons in control and experimental mice (Figure 3d, e) suggesting that if there were any changes in morphology, they were subtle. It is important to note that even subtle changes in morphology, such as changes in number of dendritic spines or differing dendritic lengths could have significant effects on the physiology of neurons and synapses. These results suggest that caffeine administration does not influence the immediate differentiation of newly born cells in the adult hippocampus. Furthermore, these results indicate that caffeine induced DNA damage or apoptosis are unlikely to explain the changes in BrdU labeling we observed. If the BrdU staining we observed were primarily due to DNA damage induced integration of BrdU, one would expect that the percentage of BrdU positive cells expressing doublecortin would differ between experimental and control mice.

3.2 Caffeine's effects on the survival of adult-born neurons

Caffeine exposure could influence the survival as well as the proliferation of hippocampal precursors. Stimuli such as voluntary running and enriched environment influence both the proliferation and survival of adult-born neurons. To assess the effects of extended caffeine administration on the survival of adult-born hippocampal neurons, we administered BrdU, treated the mice with 20 or 60 mg/kg of caffeine via twice daily injections for 28 days, and sacrificed them (Figure 1d). Neither experimental group had a significantly different number of recently born cells than controls (Figure 2d). To assess the effects of caffeine on the neuronal differentiation of adult-born cells, we quantified the percentage of BrdU positive cells expressing NeuN, a mature neuronal marker. Approximately 80% of cells differentiated into mature neurons in both control and experimental cohorts (Figure 2e, 3f–h). These results indicate that the effects of extended caffeine exposure on adult hippocampal neurogenesis are relatively specific; caffeine influences proliferation, but not neuronal differentiation or survival.

Of particular interest was assessing whether the newborn cells induced by high doses of caffeine survived, differentiated into neurons and yielded a genuine increase in neurogenesis. To this end, we treated mice with 60 mg/kg of caffeine per day for 10 days, administered BrdU twice daily on the last three days, and maintained the mice for an additional 28 or 56 days without

further caffeine treatment (Figure 1e). Experimental and control mice had similar numbers of BrdU+ cells following this experiment (Figure 2f). The surviving BrdU+ cells differentiate into mature, NeuN expressing neurons in proportions similar to controls at 28 days after treatment (Figure 2g). Although extended, high dose caffeine treatment induces a significant increase in proliferation within the hippocampus, experimental mice have no more new neurons than controls after 4 or 8 weeks.

4. Discussion

Extended treatment with caffeine influences the proliferation of precursors in the adult hippocampus in a dose dependent manner. Moderate doses of caffeine (20–30 mg/kg/day) depress proliferation, while the highest dose tested (60 mg/kg/day) increased proliferation. These results are consistent with previously reported results showing that a single dose of caffeine depressed proliferation (Han et al., 2007), and establish the dose response curve of this effect. While extrapolating effects from mice to humans presents a number of challenges, 25 mg/kg of caffeine in rodent models is generally assumed to be equivalent to 5–7 cups of coffee, or 625 mg of caffeine, in adult humans (Fredholm et al., 1999). Our results indicate that physiologically plausible doses of caffeine significantly depress hippocampal neurogenesis in a mouse model of hippocampal neurogenesis.

Caffeine has well established and extensively quantified effects on locomotor activity. The locomotor effects of caffeine become apparent at approximately three (3) mg/kg, peak at about 30 mg/kg, and depress locomotor activity at higher doses (Finn and Holtzman, 1986; Nikodijevic et al., 1993). Our gross observations are consistent with previous reports; locomotor activity was clearly increased after 10 to 30 mg/kg individual injections of caffeine. It is important to emphasize that caffeine was administered via twice daily injections. Thus, the highest dosage tested, 60 mg/kg/day, consisted of two 30 mg/kg caffeine injections, both of which increased locomotor activity.

In concordance with previous results (Han et al., 2007), we find that acute exposure to caffeine has no significant effects on the proliferation of hippocampal precursors. Acute administration of caffeine also serves as an important control to eliminate the potential confounding effects of caffeine-induced vasoconstriction on BrdU labeling. Vasoconstriction occurs within 30 minutes of administration and last for at least 90 minutes (Mathew et al., 1983; Mathew and Wilson, 1985), potentially reducing BrdU entry into the brain and yielding an artifactual reduction in BrdU labeled cells. In our experiments, however, we find that acute caffeine administration does not produce any changes in the number of BrdU labeled cells in the hippocampus, suggesting that vasoconstriction is an unlikely explanation.

One hypothesis is that caffeine's effects on neurogenesis are secondary to the longer-term effects of extended caffeine exposure, perhaps related to habituation or adaptation to caffeine. Caffeine influences a number of biochemical effects and alters the activity of cortical, thalamic, and striatal neurons; extended administration of caffeine presumably induces compensatory changes in these systems. The biochemical changes induced by caffeine administration reported in the literature, however, do not seem to involve mechanisms known to modulate proliferation. The effect of caffeine on neurogenesis takes some time to develop, temporally similar to the effects of antidepressants (Duman et al., 2001), which increase hippocampal neurogenesis. It will be interesting to explore the likely multiple mechanisms underlying "slow-onset" modifiers of neurogenesis.

Some factors that influence proliferation, such as epidermal growth factor, also limit the ability of precursors to differentiate into neurons (Kuhn et al., 1997). We tested whether caffeine administration affected the early or long term differentiation of newly generated cells in the

adult hippocampus. Newly generated cells differentiated into Dcx expressing immature neurons and NeuN expressing mature neurons at normal rates. Dcx positive cells in caffeine treated mice have grossly similar morphologies to those in control mice as well. Adenosine receptor signaling, which is antagonized by caffeine, has been shown to potentiate BDNF signaling in the hippocampus (Diogenes et al., 2007). BDNF, in turn, has been established as an important factor in modulating the survival of adult-born hippocampal neurons, and has been implicated in exercise-induced survival of adult-born neurons (Olson et al., 2006). Despite the expected reduction in BDNF activity following caffeine exposure, and in contrast to Han et al., we did not find any effect of extended caffeine treatment on the long-term survival of adult-born neurons. Our results indicate that the effects of caffeine on adult neurogenesis appear to be relatively specific.

Our pilot experiments assessing the number of Dcx positive cells following caffeine treatment yielded no significant differences in the number of Dcx positive cells after one week of 10 or 30 mg/Kg caffeine treatment. However, this is not necessarily inconsistent with the changes revealed by BrdU staining. Dcx is expressed over a broad developmental window, spanning approximately 2 weeks; Dcx is expressed by dividing cells, cells extending immature processes, as well as cells that have migrated into the GCL and exhibit morphologies consistent with maturing granule cells (Brown et al., 2003; Rao and Shetty, 2004). We speculate that the significant increase in number of Dcx positive cells, some of which were generated up to a week before caffeine treatment was even started, masked the changes to the immature, dividing fraction of the Dcx positive population.

Unexpectedly, extremely high doses of caffeine, beyond physiologically plausible dosages, increased proliferation in the hippocampus. We examined whether neurons generated in response to 60mg/kg/day of caffeine survived and contributed to a true long-term increase in neurogenesis. Four weeks after caffeine treatment, there were no more new neurons in caffeine treated mice than there were in control mice. These results suggest that caffeine induced neurons die at a faster rate than control neurons. To examine whether caffeine induced neurons continue to disappear after the control population has stabilized, we performed the same experiment but waited for eight weeks before sacrificing the mice. Control and experimental mice had similar numbers of BrdU positive cells by eight weeks after caffeine treatment. Similarly, elevating estrogen levels increases proliferation without yielding a long-lasting increase in neurogenesis (Tanapat et al., 1999) and exposure to a stressful predator scent depresses proliferation without yielding a reduction in neurogenesis (Tanapat et al., 2001). These results are consistent with the trophic factor hypothesis, which suggests that there may be a limited supply of the factors necessary for survival in the adult-hippocampus; adding new neurons beyond this level does not increase long-term neurogenesis in such paradigms. In contrast, stimuli such as enriched environment (Kempermann et al., 1997), antidepressants (Duman et al., 2001; Santarelli et al., 2003), corticosteroids (Cameron and McKay, 1999) or pheromone exposure (Mak et al., 2007) increase proliferation and yield genuine, long lasting increases in neurogenesis. Potentially, these other proliferation-inducing stimuli induce alterations in the brain that better allow the brain to integrate the newly generated adult-born neurons and sustain their survival.

Extended, but not acute caffeine treatment depresses proliferation at moderate doses and increases proliferation at high doses, suggesting that its effects are mediated through complex and potentially indirect pathways. Caffeine may function directly on A1 adenosine receptors in the dentate gyrus; however, it could also influence neurogenesis by affecting behavior. Sleep deprivation and stress depress proliferation, while increased exercise increases hippocampal proliferation. Potentially, these influences are working in opposition, the inhibitory effects dominating at moderate doses and the stimulatory effects winning out at the highest dose tested. Caffeine was administered twice daily – potentially, the morning injection could be disrupting

sleep patterns of the nocturnal mice and downregulating neurogenesis. The highest dose of caffeine tested, which increases proliferation, is also reported to result in a peak increase in locomotor activity, potentially increasing proliferation. However, caffeine could also influence neurogenesis via vasoconstrictive effects on the vascular neurogenic niche, or other as yet undiscovered mechanisms.

Physiologically plausible doses of caffeine influence the proliferation of hippocampal neural precursors in a dose and duration dependent manner, potentially influencing the circuits into which adult-born neurons integrate. The survival and differentiation of adult born neurons following caffeine administration are largely unaffected. Unexpectedly, doses of caffeine that are thought to have generally positive effects on attention and activity depress neurogenesis in the adult hippocampus. It will be particularly interesting, and potentially therapeutically useful, to examine how the multiple pathways influenced by caffeine interact to modulate hippocampal neurogenesis.

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A. Proliferation: chronic exposure

B. Proliferation: acute exposure

C. Early differentiation

D. Survival: chronic exposure

E. Survival: caffeine induced cells

Figure 1.

Methods schematic. A. Proliferation: extended exposure. Mice received twice daily injections of caffeine for 8–10 days. They received their first BrdU injection with the final injection of caffeine, their second BrdU injection 2 hrs later, and were sacrificed at 4 hours. B. Proliferation: acute exposure. Mice received 2 injections of caffeine, separated by 12 hours. They were treated with BrdU and sacrificed as in 1A. C. Mice received 60 mg/kg/day of caffeine per day for 8 days. They were treated with BrdU and maintained for another three days with continued caffeine administration. D. Mice received two injections of BrdU, separated by two hours, and were then treated with caffeine for 28 days before sacrifice. E. Mice received 30 mg/kg injections of caffeine twice daily for 8 days. They received twice daily 100 mg/kg BrdU injections on days 8, 9, and 10. They were allowed to survive for 4 or 8 weeks, and then sacrificed to assess survival and differentiation of cells born under the influence of caffeine.

Figure 2.

Caffeine alters proliferation, but not differentiation or survival, of neuronal precursors in the adult hippocampus. A. Extended caffeine administration depresses proliferation in the dentate gyrus at moderate doses and increases proliferation at high doses. Statistical results from a two tailed t-test are indicated. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.) There were 6 mice in the 10 mg/kg/day group and 5 in each of the other experimental groups. There were 3 mice in the 30 mg/kg/day control group and 4 in each of the other control groups, indicated by gray bars. The coefficients of error (CE) for caffeine and control cohorts ranged from 5.2 to 11.9%. B. Acute caffeine administration does not alter proliferation. C. Cells born following caffeine administration differentiate into doublecortin expressing neurons at normal rates. Caffeine selectively affects proliferation; survival (D) and differentiation (E) of adult-born neurons are not influenced by long-term caffeine administration. F. Increasing the number of newborn neurons does not increase the rate of long-term neurogenesis, since caffeine induced cells do not survive over 4 or 8 weeks. CE's ranged from 4.9 to 5.9% for caffeine and control cohorts in both groups. G. Cells born after extended 60 mg/kg/day caffeine treatments continue to differentiate into neurons at normal rates.

Figure 3.

Newly generated cells differentiate into neurons and adopt appropriate morphologies following caffeine administration. A–C The majority of BrdU positive (green) cells express doublecortin (red) in mice receiving extended 60 mg/kg/day caffeine treatments. Confocal stack. D, E Doublecortin expressing cells extended processes from their position in the subgranular zone through the dentate gyrus and toward the molecular layer of the dentate in both control and experimental animals. F–H Confocal microscopy confirms that adult-born neurons differentiate into mature, NeuN-expressing (red) neurons following extended caffeine administration. Scale bars: A–C, F–H 20 microns; D, E 100 microns.

Table 1

Caffeine	# BrdU+	stddev	n=	P value	t=
10 mg/kg	1068	38.3	6	0.7349	0.3421
20 mg/kg	740	14.6	5	0.0004	4.089
25 mg/kg	784	16.8	5	0.0018	3.47
30 mg/kg	841	26.9	5	0.0167	2.558
40 mg/kg	976	22.4	5	0.3988	0.8579
60 mg/kg	1606	34.9	5	0.0001	6.871
control	1041	26.3	23		