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## Brief Communication

# Systemic 5-bromo-2-deoxyuridine induces conditioned flavor aversion and c-Fos in the visceral neuraxis

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5-bromo-2-deoxyuridine (BrdU) is often used in studies of adult neurogenesis and olfactory learning, but it can also have toxic effects on highly proliferative tissue. We found that pairing Kool-Aid flavors with acute systemic injections of BrdU induced strong conditioned flavor aversions. Intermittent injections during Kool-Aid-glucose conditioning interfered with learning of a conditioned flavor-nutrient preference. Acute injection of BrdU also elevated plasma corticosterone levels and induced c-Fos in the visceral neuraxis. Thus, acute or intermittent systemic injections of BrdU (50–200 mg/kg) have aversive effects that may interfere with learning.

5-bromo-2-deoxyuridine (BrdU), a thymidine analog, incorporates into daughter DNA during the S-phase of cell division, marking new cells. BrdU is commonly used to label proliferation of new neurons in the mammalian brain during adult neurogenesis that may contribute to learning. Enhanced hippocampal neurogenesis, as labeled by BrdU, improves long-term spatial and visual recognition memory (Thuret et al. 2009). Additionally, olfactory learning impacts survival of newborn neurons (labeled with BrdU) after integration in the adult olfactory bulb (Mouret et al. 2008).

BrdU is often used in adult neurogenesis associative learning paradigms such as olfactory discrimination (as measured by habituation/disinhibition or by olfactory-cued reward task) (Mandairon et al. 2006; Mouret et al. 2008, 2009), olfactory associative learning (associating an odor with a hidden food reward) (Sultan et al. 2010), pair bonding in voles (Smith et al. 2001), and in nonassociative learning or declarative paradigms such as olfactory habituation (Magavi et al. 2005), olfactory enrichment (Rochefort and Lledo 2005; Veyrac et al. 2009), and hippocampal-dependent spatial learning and object recognition (Thuret et al. 2009; Creer et al. 2010).

A potential confound for studies of adult neurogenesis in learning and memory is the toxicity of BrdU. Systemic injections of BrdU could have aversive consequences that might alter or influence learning. Because BrdU is incorporated into rapidly dividing cells, it can be toxic to highly proliferative tissues. BrdU has well-known teratogenic effects (Franz and Kleinebrecht 1982; Kolb et al. 1999). It also has been used for cancer treatment based on toxicity to and radiosensitization of mitotically active tumor cells (Lawrence et al. 1992).

BrdU is toxic to normal tissues with high proliferation rates, such as the skin and gastrointestinal tract. For example, in clinical trials of systemic BrdU in humans, BrdU caused rashes, mucositis, nail loss, and exfoliative dermatitis (Levin et al. 1995). BrdU is incorporated heavily in the intestine as early as 1 h following i.p. injection (Kriss and Revesz 1962), suggesting possible effects on endothelial proliferation within the lining of the gut. High doses of chronic BrdU (e.g., 200 mg/kg/day for 10 d) led to weight loss in mice (Lawrence et al. 1992).

BrdU is toxic to proliferating neurons at high doses in vitro (Caldwell et al. 2005). However, doses of 100–600 mg/kg i.p. have been found to be nontoxic to proliferating neurons in the hippocampus of adult rats (Cameron and McKay 2001). Nonetheless, BrdU might have aversive effects secondary to toxic effects on proliferative tissues in the periphery, such as in the gastrointestinal tract.

To determine whether BrdU has toxic effects that would impact behavior and neural processing, we assessed conditioned flavor aversion (CFA), interference with conditioned nutrient-flavor preference (CFNP) learning, corticosterone release, and c-Fos following systemic injections of BrdU. We used doses of BrdU that are standard for visualization of proliferating neurons (i.e., 50–200 mg/kg).

CFA is a form of associative learning in which an animal associates a novel flavor or taste with an aversive consequence, and subsequently avoids ingesting the flavor. CFA is a sensitive assay, such that a CFA can be observed in the absence of any other apparent toxic effect (Gamzu et al. 1985). The formation of a robust CFA often requires only one conditioning trial (Garcia and Kimeldorf 1957). Drugs and treatments with antimitotic activity can serve as potent aversive stimuli. Radiation (Garcia et al. 1955), cisplatin (Rudd et al. 1998), and cyclophosphamide (Mungarndee et al. 2006) all cause nausea and malaise in humans and are effective as a US in CFA paradigms.

To examine whether systemic BrdU could lead to a formation of a CFA, we paired ingestion of Kool-Aid/saccharin with a single i.p. injection of BrdU. Adult male rats ( $n = 24$ ) were placed on a water restriction schedule. On conditioning day, rats were allowed a 10-min access to 0.05% Kool-Aid mixed with 0.05% saccharin (counterbalanced, cherry and grape; CS+). Rats drank an average of  $5.2 \pm 0.3$  g, with no difference in intake among groups. Five min after access to the CS+ rats were divided into four groups and injected with one of four solutions: vehicle (0.007 N NaOH, 0.15 M NaCl, 20 mL/kg), a low dose of BrdU (50 mg/kg), a high dose of BrdU (200 mg/kg), or LiCl (0.15 M, 76 mg/kg) as a positive control. Following injections, water was returned overnight. The next day, two-bottle preference testing was begun with CS+ and an unconditioned Kool-Aid/saccharin (CS-) solution and continued for 14 d after conditioning. Preference score was calculated as CS+ intake over total intake of CS+ and CS-. Vehicle-injected rats were predicted to show a 50% preference for the CS+ flavor over the CS- flavor; lower CS+ preference would indicate acquisition of a CFA.

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During two-bottle preference testing, the vehicle and low dose BrdU groups showed a preference close to 50%, indicating equal intakes of CS+ and CS-flavors. The LiCl and high-dose BrdU groups drank almost none of the CS+ flavor, indicating low preference and a strong CFA (Fig. 1). Two-way ANOVA revealed a significant effect of groups ( $F_{(3,20)} = 20.35$ ,  $P < 0.00001$ ), but no effect of days and no interaction. Post-hoc Newman Keuls revealed no significant difference between the low-dose BrdU and vehicle groups. Both the high-dose and LiCl groups displayed significantly lower CS+ preferences than the vehicle group, and there was no significant difference in CS+ preference between the high dose BrdU and LiCl groups. Thus, the high dose BrdU and LiCl groups formed equivalent CFAs.

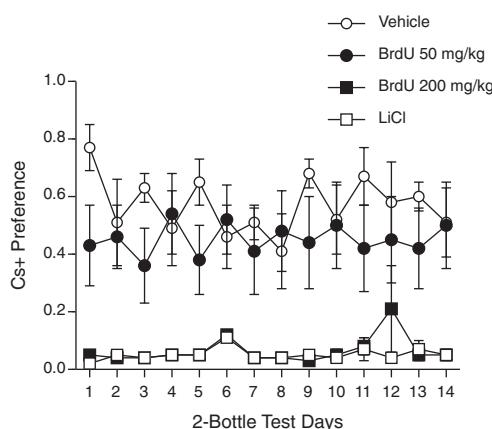
Although a single injection of 50 mg/kg BrdU failed to induce a CFA, multiple pairings of a CS and US can lead to a stronger CFA (Riley and Mastropaoletti 1989). Therefore, we examined whether low-dose BrdU could induce a CFA after multiple pairings by giving three consecutive days of conditioning pairing Kool-Aid with 50 mg/kg injections of BrdU.

Rats ( $n = 12$ ) were placed on water restriction and then conditioned on three consecutive days. On each conditioning day rats were allowed a 10-min access to 0.05% Kool-Aid mixed with 0.05% saccharin (CS+, counterbalanced cherry and grape). Rats were injected with either vehicle or BrdU (50 mg/kg) 5-min following CS+ access. The day after the third conditioning day, expression of CFA was examined for the 6-d via two-bottle preference testing with the CS+ and CS-solutions.

Two-way ANOVA on conditioning intakes across the 3 d of conditioning showed that there was an effect of days ( $F_{(2,20)} = 28.12$ ,  $P < 0.00001$ ), but not group and no interaction. Intake of the BrdU group was significantly lower than the vehicle group on day 2 of conditioning, but not on days 1 or 3 (data not shown).

During two-bottle preference testing, neither the BrdU nor vehicle groups showed an overall average preference lower than 50%. There was a main effect of group ( $F_{(1,10)} = 9.81$ ,  $P < 0.05$ ), but no effect of days and no interaction. Therefore, intakes across the 6 d of preference testing were pooled for group comparison alone. The average CS+ preference of the BrdU group was significantly lower than the CS+ preference of the vehicle group ( $51 \pm 3\%$  vs.  $69 \pm 5\%$ ;  $P < 0.01$ ).

Although BrdU induced a CFA when explicitly paired with a novel flavor, BrdU is often administered across several days during



**Figure 1.** Preference for a Kool-Aid flavor (CS+) paired with drug treatment over an unpaired Kool-Aid flavor (CS-) across 14 d of two-bottle tests. After one pairing of the CS+ with either 200 mg/kg BrdU (black squares) or LiCl (white squares), rats showed a significantly lower preference compared with vehicle-treated rats (white circles). CS+ preference in rats treated with 50 mg/kg BrdU (black circles) was not significantly lower than in vehicle-treated rats.

a distinct learning protocol to assess learning-associated neurogenesis. A typical neurogenesis study using BrdU injections often involves multiple injections over the course of a 24- to 48-h period. Multiple injections allow labeling of cells that are going through S-phase divisions across the day, and thus incorporating BrdU at different times (Cameron and McKay 2001). These studies often result in a total dose of BrdU ranging from 100 mg/kg to 300 mg/kg per day. If BrdU has aversive effects, then a typical neurogenesis protocol of BrdU administration might interfere with the learning under study.

To determine whether BrdU can disrupt learning, we administered BrdU concurrently with CFNP acquisition. CFNP learning is a robust form of olfactory learning in which a flavor is paired with nutrient content (e.g., glucose), producing a strong preference for the nutrient-paired flavor over non-nutritive flavors (Ackroff and Sclafani 1991; Sclafani et al. 1993; Ackroff et al. 2009). Disruption of CFNP learning by BrdU injections would indicate an alteration of learning following BrdU injection.

Rats ( $n = 16$ ) were assigned to two groups of eight. One group received vehicle injections, while the other group received BrdU injections (100 mg/kg). Injections were given at 2 h after lights off and repeated every 12 h for a total of four injections over 48 h. Thus, rats received intermittent injections of BrdU at a dose that is not neurotoxic (Cameron and McKay 2001).

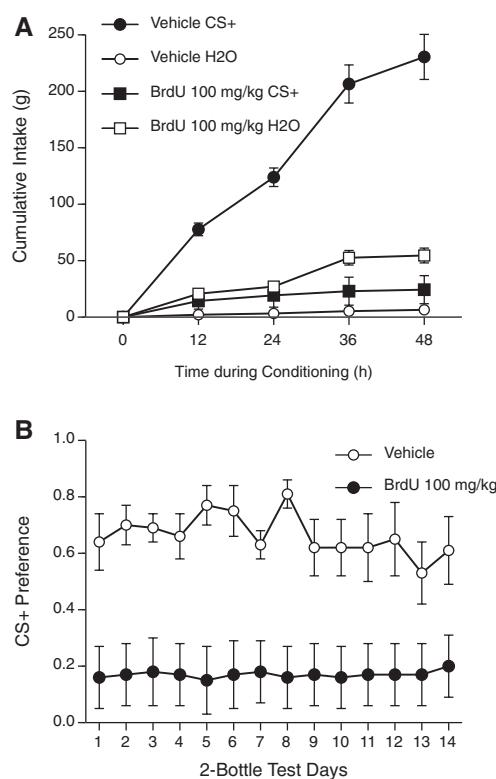
Immediately following the first injection, rats were given 48-h access to two bottles: a solution of 8% glucose, 0.05% sodium saccharin, and 0.05% Kool-Aid (CS+, grape or cherry counterbalanced); and water. Intake was measured every 12 h. At 24 h, solutions were refilled and bottle positions were switched. Following 48 h of conditioning, two-bottle preference testing with both CS+/saccharin (without glucose) and unconditioned Kool-Aid/saccharin (CS-) solutions was begun and continued for 14 consecutive days.

During 48-h conditioning the cumulative intake of CS+ by vehicle-treated rats increased over the course of 48 h; water intake remained low. The BrdU-treated rats had slightly increased intake of water over 48 h, while the CS+ intake remained low (Fig. 2A). There were significant effects of group and hour during conditioning and an interaction ( $F_{(3,42)} = 73.56$ ,  $P < 0.0001$ ), such that the CS+ intake of the BrdU group was significantly lower than the vehicle group at all time points.

During two-bottle preference testing there was an effect of group ( $F_{(1,14)} = 18.86$ ,  $P < 0.001$ ) with a significant difference between BrdU and vehicle-treated rats on all days (Fig. 2B). Thus, concurrent administration of BrdU during CNFP conditioning prevented a CNFP from forming and instead led to CFA acquisition.

We also determined whether BrdU had acute behavioral or physiological effects. After injection of a malaise-inducing toxin, rats display "lying-on-belly" (LOB) by maintaining an extended prone posture on the cage floor (Bernstein et al. 1992). Six rats were observed for LOB for 1 h following i.p. injection of vehicle, BrdU (200 mg/kg), or LiCl (76 mg/kg). Every rat received each treatment counterbalanced across days, with at least 3 d between injections. LiCl injections caused LOB in all rats (6/6) with an average latency of  $18 \pm 2$  min, while LOB after BrdU was observed at 46 min post-injection in one rat (1/6). LOB was not observed after vehicle injections.

Although we did not observe an acute behavioral effect, we examined corticosterone levels at 1 h after BrdU injection. LiCl injection elevates plasma corticosterone in rats (Hennessy et al. 1976; Smotherman et al. 1976; Spencer et al. 2005) as does cyclophosphamide (Di Renzo et al. 1977). Rats ( $n = 12$ /group) received an i.p. injection of vehicle or BrdU (200 mg/kg). One hour later rats were anesthetized with CO<sub>2</sub>, decapitated, and trunk blood was collected. Plasma was processed for rat corticosterone by



**Figure 2.** (A) Cumulative intake of Kool-Aid flavor mixed with glucose (CS+, black symbols) or water (white symbols) by rats injected with vehicle (circles) or BrdU (100 mg/kg, squares) at 12-h intervals across a 48-h conditioning period. Vehicle-treated rats showed robust intake of the CS+ with almost no water consumption; BrdU-treated rats showed a preference for water over the CS+. (B) Preference for the CS+ flavor mixed with saccharin over an unpaired flavor (CS-) across 14 d of two-bottle preference tests. Vehicle-treated rats (white circles) showed a strong preference for the CS+, while BrdU-treated rats (black circles) showed an aversion toward the CS+.

radioimmunoassay (Coat-A-Count, Siemens). BrdU caused a significant increase ( $P < 0.05$ ) in plasma corticosterone levels ( $114.6 \pm 27.8$  ng/mL) compared with vehicle ( $41.2 \pm 10.9$  ng/mL).

Finally, we explored whether BrdU would induce c-Fos expression patterns in the visceral neuraxis comparable to other toxins. We examined c-Fos in the visceral neuraxis at 1 and 3 h after BrdU injection in the nucleus of the solitary tract (NTS), the parabrachial nucleus (PBN), and the central amygdala (CeA).

Rats were injected with vehicle ( $n = 4$ /time point) or with BrdU ( $n = 4$ /time point, 200 mg/kg). At 1 and 3 h after injection, rat brains were removed and processed for c-Fos immunoreactivity as previously described (Kwon et al. 2008).

At 1 h after injection there was significantly more c-Fos expression in BrdU- vs. vehicle-treated rats in the NTS ( $7.4 \pm 1.8$  vs.  $2.8 \pm 0.9$ ,  $P < 0.05$ ) but not the PBN or CeA. At 3 h after injection there was significantly greater c-Fos expression in BrdU- vs. vehicle-treated rats in the NTS ( $15.3 \pm 5.8$  vs.  $3.1 \pm 0.6$ ;  $P < 0.05$ ) and CeA ( $26.2 \pm 8.3$  vs.  $5.7 \pm 1.3$ ;  $P < 0.05$ ), but not in the PBN ( $11.8 \pm 4.3$  vs.  $4.0 \pm 1.0$ ;  $P < 0.07$ ) (Fig. 3). Much of the c-Fos in the NTS region was located near the border of the NTS and area postrema (AP), although very little c-Fos was seen in the AP itself. In the PBN, c-Fos was localized to the lateral and external subnuclei.

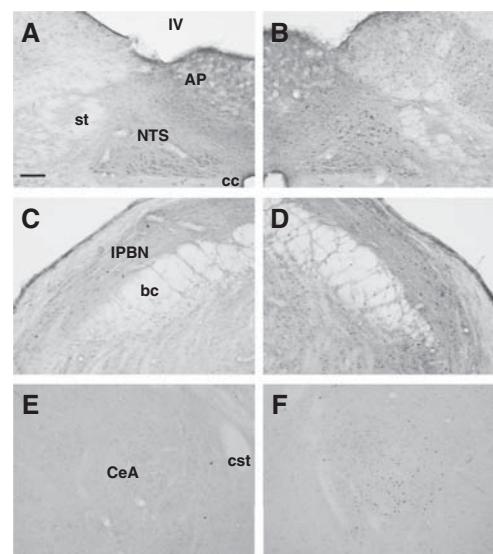
BrdU activation of the visceral neuraxis is consistent with an effect on the gastrointestinal tract and visceral afferents (e.g., the vagus nerve) or chemoreceptors of the area postrema (Bernstein et al. 1992; Saper 2002). For example, c-Fos response in the brain

following LiCl injection occurs heavily in the NTS, PBN and CeA (Yamamoto et al. 1992; Swank and Bernstein 1994; Spencer and Houpt 2001; Rinaman and Dzmitra 2007). Cisplatin induces similar c-Fos expression in the NTS and AP, which relies on vagal input (Horn et al. 2004, 2007; Horn 2009). Thus, expression of c-Fos in the visceral neuraxis is an indicator of visceral stimulation.

In conclusion, these results demonstrate that systemic BrdU has aversive effects on rats even in the absence of observable behavioral effects. Systemic injections of a high dose of BrdU (200 mg/kg, i.p.) paired with a flavor induced a profound CFA, while a lower dose (50 mg/kg) had a milder aversive effect after multiple pairings. Furthermore, BrdU administration (as in a typical adult neurogenesis paradigm) during acquisition of an olfactory-based CFNP was disruptive to the learning. Additionally, we showed that injections of BrdU stimulated corticosterone release and increased c-Fos expression in the NTS and CeA, suggesting that BrdU treatment activates the hypothalamic-pituitary-adrenal axis and visceral neuraxis in parallel with its aversive effects.

We have not identified the mechanism of BrdU's aversive action. BrdU is incorporated into mitotically active cells, and can therefore have a cytotoxic effect on rapidly dividing tissues (Caldwell et al. 2005). Thus, BrdU could have an effect similar to other treatments that damage proliferating cells (e.g., whole-body irradiation (Garcia et al. 1955), cisplatin (Rudd et al. 1998), or cyclophosphamide (Mungarndee et al. 2006)). The gastrointestinal tract, with rapid turnover of cells, shows a high rate of BrdU incorporation (Kriss and Revesz 1962), and thus is a potential site of BrdU action that could lead to aversive effects and CFA acquisition.

The doses of BrdU typically used in studies of adult neurogenesis are above the threshold required for aversive effects (Cameron and McKay 2001). Thus, there is the potential for aversive effects of BrdU to interact with or confound learning. Others have also shown that BrdU may interfere with learning (Komissarova et al. 2010). Olfactory learning may be especially



**Figure 3.** Photomicrographs of c-Fos expression 3 h after injection of vehicle (A,C,E) or BrdU (200 mg/kg; B,D,F) in the NTS (A,B), PBN (C,D), and CeA (E,F). Scale bar in A: 100 microns. (IV) Fourth ventricle; (cc) central canal; (st) solitary tract; (AP) area postrema; (NTS) nucleus of the solitary tract; (bc) brachium conjunctivum; (IPBN) lateral parabrachial nucleus; (cst) commisural stria terminalis; (CeA) central nucleus of the amygdala.

susceptible to aversive interference, given the ease in which odor or flavor aversions can be acquired. Stimuli producing visceral toxic effects can be strongly associated with flavor, taste, and ingestion; other exteroceptive stimuli may not be as strongly associated with the aversive effects of BrdU injections (Garcia and Ervin 1968).

BrdU injections may disrupt other types of learning due to elevated corticosterone levels, such as disruption of hippocampal-dependant spatial memory by acute stress (Cazakoff et al. 2010). Furthermore, BrdU would most likely interfere with learning when administered during the acquisition period. There is no reason to believe prior administration of BrdU (i.e., to label mature neurons that proliferated days or weeks earlier) would interfere with subsequent learning.

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