

RESEARCH PAPER

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# Cannabinoid receptor signaling induces proliferation but not neurogenesis in the mouse olfactory epithelium

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## ABSTRACT

The olfactory epithelium actively generates neurons through adulthood, and this neurogenesis is tightly regulated by multiple factors that are not fully defined. Here, we examined the role of cannabinoids in the regulation of neurogenesis in the mouse olfactory epithelium. *In vivo* proliferation and cell lineage studies were performed in mice (C57BL/6 and cannabinoid type 1 and 2 receptor deficient strains) treated with cannabinoids directly (WIN 55,212–2 or 2-arachidonylglycerol ether) or indirectly via inhibition of cannabinoid hydrolytic enzymes. Cannabinoids increased proliferation in neonatal and adult mice, and had no effect on proliferation in cannabinoid type 1 and 2 receptor deficient adult mice. Pretreatment with the cannabinoid type 1 receptor antagonist AM251 decreased cannabinoid-induced proliferation in adult mice. Despite a cannabinoid-induced increase in proliferation, there was no change in newly generated neurons or non-neuronal cells 16 d post-treatment. However, cannabinoid administration increased apoptotic cell death at 72 hours post-treatment and by 16 d the level of apoptosis dropped to control levels. Thus, cannabinoids induce proliferation, but do not induce neurogenesis nor non-neuronal cell generation. Cannabinoid receptor signaling may regulate the balance of progenitor cell survival and proliferation in adult mouse olfactory epithelium.

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cell death; cannabinoid receptor 1 and 2 deficient mice; endocannabinoids; olfactory system; progenitor cells; proliferation

## Introduction

Adult neurogenesis in the mammalian brain is continuous throughout life and contributes to the plasticity and the potential for repair of the central nervous system. Two regions in the brain, the subventricular zone of the lateral ventricles and the subgranular zone of the dentate gyrus in the hippocampus, consistently undergo adult neurogenesis under physiological conditions.<sup>1–3</sup> In addition, the peripheral olfactory epithelium actively generates neurons throughout adulthood.<sup>4</sup> The olfactory epithelium is the only site where the primary sensory neuron is directly exposed to the external environment and is therefore in contact with airborne irritants and toxicants in addition to volatile odorants. The olfactory epithelium adapts to this harsh environment by routinely replacing olfactory sensory neurons through a population of basal progenitor cells residing in the basal portion of the epithelium just above the basement membrane.<sup>5–8</sup> The natural turn-over rate of all cells in the olfactory

epithelium (neurons, glial-like sustentacular cells, microvillous cells and basal progenitor cells) can be further increased after exposure to mechanical damage or toxicants.<sup>6,9–11</sup> Cell proliferation and neuronal differentiation in the olfactory epithelium is tightly regulated by multiple signals produced in the basal “stem” cell microenvironment, which is complex and remains to be fully defined.<sup>12</sup>

The cannabinoid system has many functions including modulating proliferation in areas of adult neurogenesis and providing neuroprotection. In the central nervous system, cannabinoid type 1 (CB1) and cannabinoid type 2 (CB2) receptors modulate neural stem cell proliferation *in vitro*<sup>13,14</sup> and *in vivo*.<sup>15–18</sup> Furthermore, in CB1 receptor deficient mice there is nearly a 50% reduction in 5'-bromo-deoxyuridine (BrdU) positive cells in the subventricular zone and dentate gyrus.<sup>17</sup> Cannabinoids are also neuroprotective in a variety of neuronal injury models. In a closed head injury mouse model,

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increasing the levels of endogenous cannabinoid 2-arachidonylglycerol (2-AG) decreased hippocampal cell death and infarct volume, and this effect was dose-dependently attenuated by a specific CB1 receptor antagonist.<sup>19</sup> The cannabinoid system has been extensively studied in the central areas of adult neurogenesis and the data collectively indicate a clear role for CB receptor signaling in progenitor cell proliferation throughout the lifespan of the animal.

The cannabinoid system is also present in central and peripheral olfactory regions. Cannabinoid receptors have been identified in rodent olfactory bulb.<sup>20</sup> Blocking cannabinoid synthesis or inhibiting CB2 receptors decreases cell proliferation in the subventricular zone and subsequent migration along the rostral migratory stream leading to reduced numbers of cells in the olfactory bulb.<sup>21,22</sup> A cannabinoid system identified in the peripheral olfactory epithelium of *Xenopus laevis* modulates odorant-evoked changes in olfactory receptor neurons.<sup>23</sup> In the same animal model, 2-AG is synthesized in both olfactory sensory neurons and glial-like sustentacular cells.<sup>24</sup> We recently discovered a cannabinoid system in the mouse olfactory epithelium<sup>25</sup> that consisted of CB1 receptors on neurons, sustentacular cells, microvillous cells and basal progenitor cells. The presence of CB2 receptor protein in the olfactory epithelium is likely due to the presence of CB2 receptors on immune cells located in the lamina propria, and not in the epithelium. Unfortunately, we were unable to confirm this using a CB2 receptor antibody specifically for immunohistochemistry due to problems with the specificity.<sup>26-28</sup> The endocannabinoid 2-arachidonylglycerol and its synthetic and degradation enzymes (diacylglycerol lipase and monoacylglycerol lipase) are present, but not *N*-arachidonylethanolamide. We did not observe a neuromodulatory role for cannabinoids in olfaction in the mouse, as demonstrated in the developing tadpole.<sup>23</sup> However, in CB1 and CB2 receptor deficient (CB1<sup>-/-</sup>/CB2<sup>-/-</sup>) mice the number of basal progenitor cells and neurons is significantly reduced, suggesting that CB receptor signaling contributes to the survival and regulation of the basal cell population.<sup>25</sup> Here, we further examined the role of cannabinoids and the CB1 receptor in neurogenesis in the mouse olfactory epithelium.

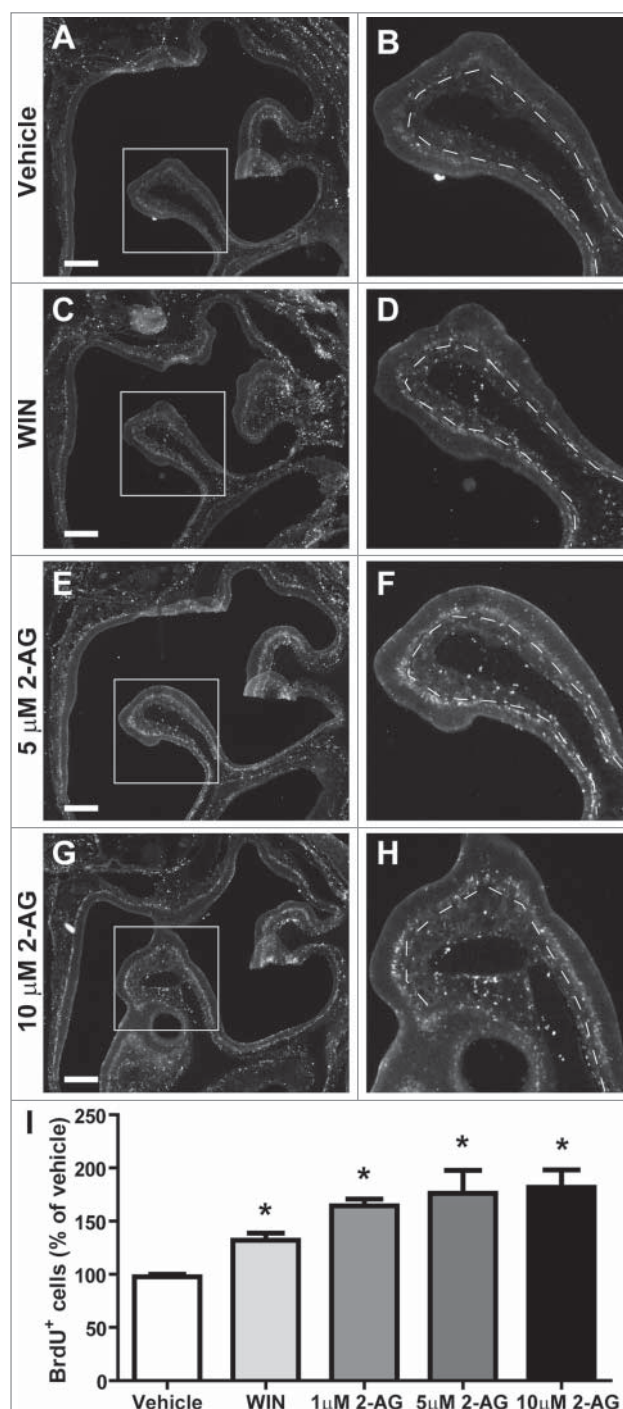
## Results

### **Exogenous and endogenous cannabinoids increase proliferation in neonatal olfactory epithelium**

To determine if cannabinoids act as a proliferative factor throughout development, BrdU incorporation was first assessed in neonatal mouse olfactory epithelium following intranasal administration of exogenous and endogenous cannabinoids. Initial proliferation experiments were done in neonates because they exhibit a high level of proliferation<sup>29,30</sup> and cannabinoid signaling strongly influences proliferation during early postnatal periods in the central nervous system.<sup>31,32</sup> Neonatal Swiss Webster mice intranasally aspirated vehicle (50% ethanol), the synthetic CB1 and CB2 receptor agonist WIN (10  $\mu$ M) or 2-AG ether (1  $\mu$ M, 5  $\mu$ M, 10  $\mu$ M) and levels of BrdU incorporation were measured 48 hours after treatment. As expected, the number of cells incorporating BrdU in the vehicle-treated neonates was high (103.4  $\pm$  7.8 cells/mm olfactory epithelium). Intranasal instillation of WIN significantly increased BrdU positive cells in the olfactory epithelium by 35  $\pm$  7% above vehicle (Fig. 1A–D, I;  $p < .05$ ,  $n = 3-4$  animals/group). Intranasal treatment with increasing concentrations of 2-AG ether significantly increased BrdU incorporation compared to vehicle by 68  $\pm$  6%, 81  $\pm$  22%, and 86  $\pm$  17%, respectively (Fig. 1E–I,  $p < .001$ ,  $n = 3-4$  animals/group). These data indicate that both exogenous and endogenous cannabinoids can promote proliferation in the neonatal mouse olfactory epithelium.

### **CB1 receptor signaling promotes proliferation in the olfactory epithelium of adult Swiss Webster mice**

Next, CB1 receptor-mediated proliferation in the adult olfactory epithelium was examined in the outbred Swiss Webster strain of mice. Mice intranasally aspirated vehicle (1% DMSO), CB1 receptor specific antagonist AM251 (10  $\mu$ M), or WIN (10  $\mu$ M) and BrdU-incorporation was quantified in the olfactory epithelium 48 hours post-administration. WIN significantly increased the number of BrdU positive cells by 27  $\pm$  1% compared to vehicle control in the olfactory epithelium of Swiss Webster mice (Fig. 2A–B, E; 27.0  $\pm$  1.6 vs. 21.2  $\pm$  1.1 cells/mm olfactory epithelium, \* $p < .01$ ). The majority of BrdU-immunoreactive cells were observed in the basal cell layer adjacent to the basement membrane, however, in some instances,



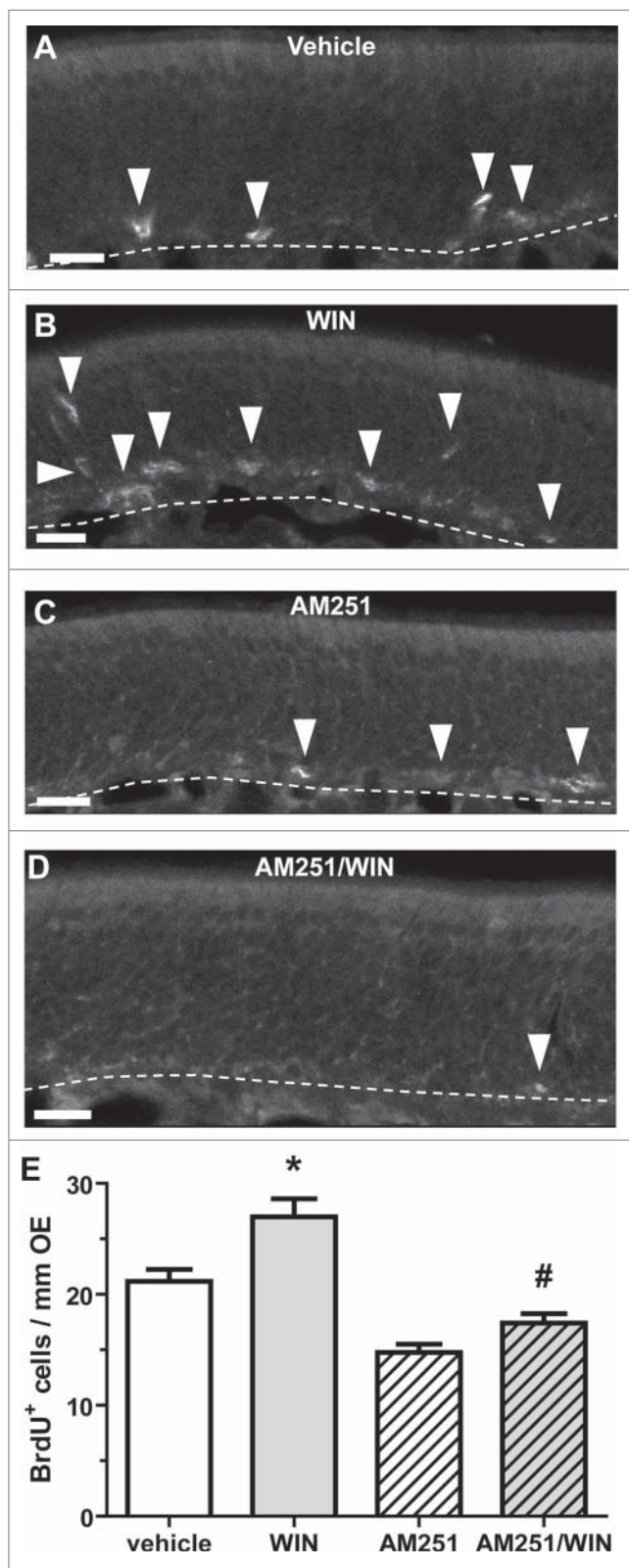
**Figure 1.** Exogenous and endogenous cannabinoids increase proliferation in neonatal olfactory epithelium. Neonatal Swiss Webster mice were intranasally administered vehicle (50% ethanol), WIN (10  $\mu$ M), or 2-AG ether (1  $\mu$ M, 5  $\mu$ M, or 10  $\mu$ M). BrdU was given (i.p., 18 mg/kg total) 2 and 4 hrs prior to tissue collection at 48 hours post-treatment. (A–H) Representative images of (A–B) vehicle control, (B–C) WIN, (E–F) 5  $\mu$ M 2-AG, and (G–H) 10  $\mu$ M 2-AG treatment groups. White boxes in A,C,F,G indicate the field of view shown in B,D,F,H, respectively. Scale bar, 100  $\mu$ m. (I) Bar graph showing normalized BrdU incorporation (mean  $\pm$  SEM). \*,  $p < 0.05$  WIN vs. vehicle,  $p < 0.001$  2-AG vs. vehicle (one-way ANOVA followed by the Bonferroni multiple comparison test;  $n = 3$ –4 mice/group).

immunoreactivity was observed in the apical or neuronal layers (e.g., Fig. 2B; see also Fig. 5). Pre-treatment with CB1 receptor-specific antagonist AM251 decreased the number of BrdU positive cells in the olfactory epithelium compared to vehicle-treated mice (Fig. 2C, E;  $14.8 \pm 0.8$  vs.  $21.2 \pm 1.1$  cells/mm olfactory epithelium; \*,  $p < 0.01$ ), suggesting there may be tonic release of endogenous cannabinoids. Moreover, AM251 pre-treatment significantly blocked the WIN-induced increase in BrdU incorporation (Fig. 2C–E;  $17.4 \pm 0.9$  vs.  $27.0 \pm 1.6$  cells/mm olfactory epithelium; #,  $p < 0.001$ ), indicating that CB1 receptors are involved in the WIN-induced increase in cell proliferation in the adult olfactory epithelium.

### Endogenous cannabinoids increase proliferation in the olfactory epithelium of adult C57BL/6 mice

Next, C57BL/6 and  $CB1^{-/-}/CB2^{-/-}$  mice intranasally aspirated vehicle (1% DMSO), WIN (10  $\mu$ M), AM251 (10  $\mu$ M), or AM251 30 minutes prior to WIN. In addition, the concentration of endogenous cannabinoids was increased by administering a mixture of JZL184 and URB597 (JZL/URB, 10  $\mu$ M and 100  $\mu$ M, respectively) to inhibit enzymes that hydrolyze 2-AG and *N*-arachidonylethanolamide. Basal cell proliferation was quantified by measuring BrdU incorporation in the olfactory epithelium 48 hours post-administration. Similar to Swiss Webster mice, WIN-treated C57BL/6 mice showed a significant  $57 \pm 11\%$  increase in BrdU incorporation over vehicle treated mice (Fig. 3A–D, M;  $49.6 \pm 3.5$  vs.  $31.6 \pm 1.1$  cells/mm olfactory epithelium;  $p < 0.01$ ). The WIN-induced increase in proliferation was reduced by CB1 receptor specific antagonist AM251 (Fig. 3E–H, M;  $49.6 \pm 3.5$  vs.  $34.4 \pm 3.7$   $p < 0.05$ ). Additionally, endogenous cannabinoid hydrolytic enzyme inhibitors JZL/URB increased the number of BrdU-positive cells over vehicle control by  $109 \pm 11\%$  (Fig. 3I–J, M;  $31.6 \pm 1.1$  vs.  $65.9 \pm 3.6$  cells/mm olfactory epithelium;  $p < 0.001$ ), suggesting that endogenous cannabinoids increase progenitor cell proliferation in the olfactory epithelium. Furthermore, JZL/URB treatment further increased BrdU incorporation compared to the WIN treatment group ( $49.6 \pm 3.5$  vs.  $65.9 \pm 3.6$  cells/mm olfactory epithelium;  $p < 0.05$ ), indicating that endogenous cannabinoids are more effective at increasing basal cell proliferation than exogenous synthetic cannabinoids at the current concentrations. In

contrast to the Swiss Webster strain, there was a slight but insignificant decrease in BrdU incorporation in the AM251 treated animals compared to vehicle control (Fig. 3M;  $24.8 \pm 1.8$  vs.  $31.6 \pm 1.1$  cells/mm

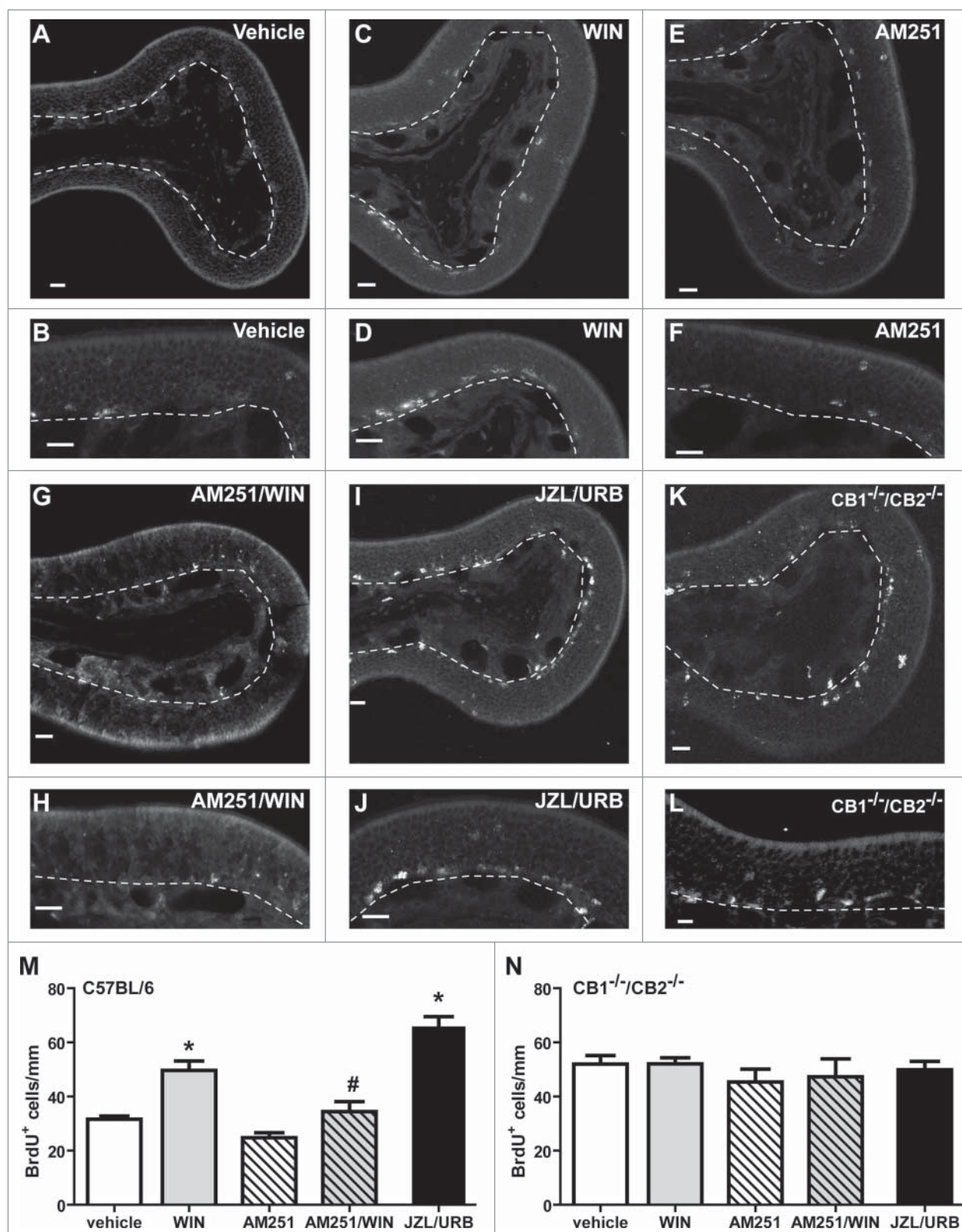


olfactory epithelium). This suggests that tonic release of endogenous cannabinoids and subsequent CB1 receptor signaling is not primarily responsible for baseline levels of proliferation in the C57BL/6 mouse olfactory epithelium, and highlights the differences in mouse strains. As expected,  $CB1^{-/-}/CB2^{-/-}$  mice showed no change in BrdU incorporation in response to any cannabinoid pharmacological manipulation. (Fig. 3K–I, N).

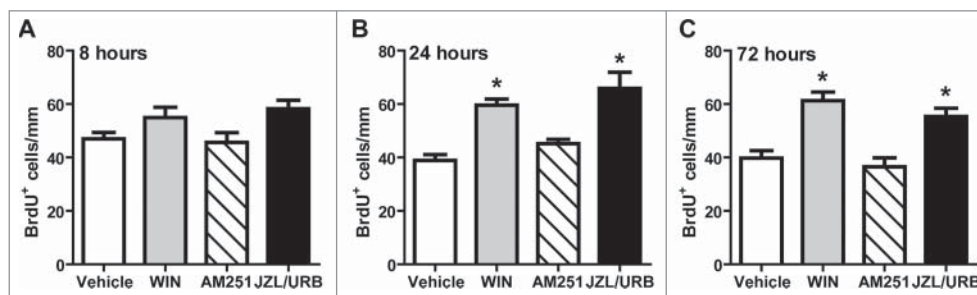
### Cannabinoids increase proliferation up to 3 d after administration

To better characterize the time course of cannabinoid-induced proliferation in the mouse olfactory epithelium, C57BL/6 mice intranasally aspirated vehicle (1% DMSO), WIN (10  $\mu$ M), AM251 (10  $\mu$ M), or JZL/URB (10  $\mu$ M and 100  $\mu$ M, respectively), and BrdU-incorporation was quantified in the olfactory epithelium 8, 24 and 72 hours post-administration. No changes in BrdU positive cells were detected across treatments at 8 hours (Fig. 4A). Twenty-four hours after intranasal aspiration, a  $53 \pm 6$  % increase in BrdU+ cells was detected in WIN-treated C57BL/6 mice compared to vehicle (Fig. 4B;  $59.5 \pm 2.3$  vs.  $38.9 \pm 2.2$ ;  $p < 0.001$ ), and a  $70 \pm 16$  % increase in JZL/URB treated mice (Fig. 4B;  $65.9 \pm 6.1$  vs.  $38.9 \pm 2.2$ ;  $p < 0.001$ ). This robust increase in proliferation was still detectable 72 hours after administration with a  $54 \pm 8$  % increase after WIN treatment compared to vehicle (Fig. 4C;  $61.2 \pm 3.3$  vs.  $39.8 \pm 2.7$ ,  $p < 0.001$ ) and a  $39 \pm 8$  % increase after JZL/URB treatment compared to vehicle (Fig. 4C;  $55.3 \pm 3.2$  vs.  $38.9 \pm 2.2$ ;  $p < 0.001$ ). No change in BrdU-incorporation was seen at 24 or 72 hours after AM251 treatment vs. vehicle (Fig. 4B–C; 24 hours  $45.2 \pm 1.6$  vs.  $38.9 \pm 2.2$ , and 72 hours  $36.5 \pm 3.3$  vs.  $39.8 \pm 2.7$ ). Overall, these

**Figure 2.** WIN promotes proliferation in the adult mouse olfactory epithelium via CB1-receptors. (A–D) Representative images of BrdU immunoreactivity in olfactory epithelium of Swiss Webster mice treated with (A) vehicle (1% DMSO), (B) CB1/CB2 receptor agonist WIN (10  $\mu$ M) (C) CB1 receptor antagonist AM251 (10  $\mu$ M), or (D) AM251 30 minutes prior to WIN (AM251/WIN). BrdU (i.p., 18 mg/kg) was injected 3 and 6 hours before tissue collection 48 hours post-treatment. Arrow heads indicate BrdU positive cells. Dashed white lines delineate basement membrane. Scale bar, 10  $\mu$ m. (E) Bar graph showing number of BrdU+ cells per each treatment group (mean  $\pm$  SEM). \* $p < 0.01$  vs. vehicle; #  $p < 0.001$  vs. WIN. (One way ANOVA followed by Bonferroni multiple comparison test;  $n = 6$  mice/group).



**Figure 3.** Endogenous cannabinoids promote proliferation in C57BL/6 but not CB1<sup>-/-</sup>/CB2<sup>-/-</sup> mice. Adult (6–8 week) male C57BL/6 and CB1<sup>-/-</sup>/CB2<sup>-/-</sup> mice (non-littermates), intranasally aspirated vehicle (1% DMSO), CB1 receptor antagonist AM251 (10  $\mu$ M), CB1/CB2 receptor agonist WIN (10  $\mu$ M), JZL/URB (10  $\mu$ M, 100  $\mu$ M, respectively), or AM251 30 minutes prior to WIN (AM251/WIN). BrdU was injected (i.p., 18 mg/kg) 3 and 6 hours before tissue collection 48 hours post-instillation. (A–L) Representative images of BrdU immunoreactivity in the endoturbinate II of C57BL/6 mice treated with (A–B) vehicle, (C–D) WIN, (E–F) AM251, (G–H) AM251/WIN, (I–J) JZL/URB. BrdU immunoreactivity in CB1<sup>-/-</sup>/CB2<sup>-/-</sup> mice treated with vehicle (K–L) is representative of BrdU immunoreactivity of all treatment groups (data not shown). Dashed white lines delineate basement membrane. Scale bar, 20  $\mu$ m. (M–N) Quantification of BrdU positive cells (mean  $\pm$  SEM) in ectoturbinate 2 and endoturbinate II of (M) C57BL/6 mice and (N) CB1<sup>-/-</sup>/CB2<sup>-/-</sup> mice. \*,  $p < 0.01$  WIN vs. vehicle,  $p < 0.001$  JZL/URB vs. vehicle; #  $p < 0.05$  vs. WIN (One way ANOVA followed by Bonferroni multiple comparison test;  $n = 4–7$  mice/group).



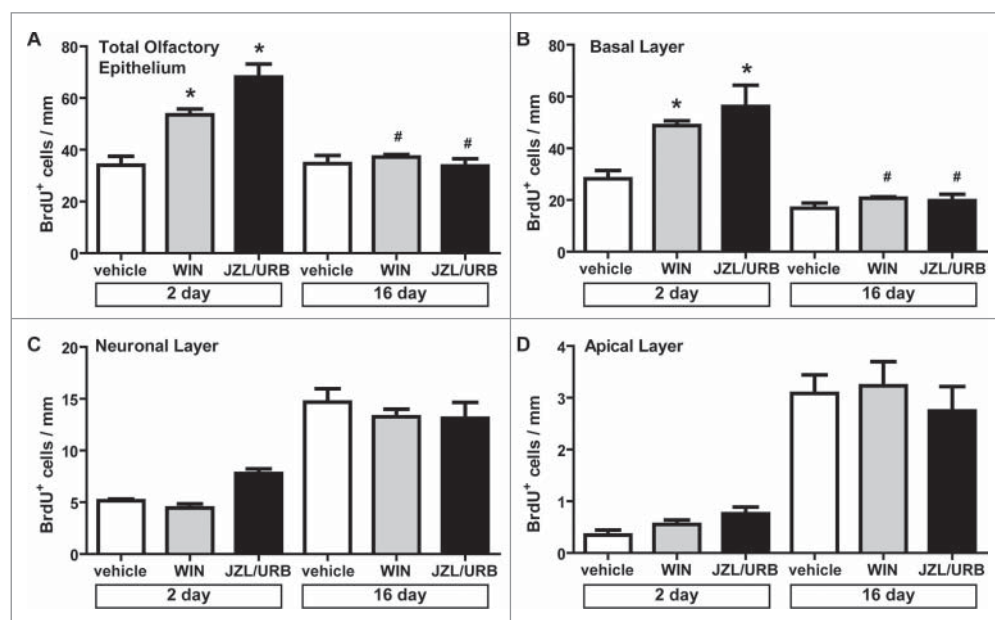
**Figure 4.** Cannabinoid-induced proliferation is detected up to 72 hours after administration. Adult (6–8 week) male C57BL/6 mice intranasally aspirated vehicle (1% DMSO), CB1 receptor antagonist AM251 (10  $\mu$ M), CB1/CB2 receptor agonist WIN (10  $\mu$ M), or JZL/URB (10  $\mu$ M, 100  $\mu$ M, respectively), and BrdU (i.p., 18 mg/kg) was injected 3 and 6 hours before tissue collection 8, 24, and 72 hours post-institution. (A–C) Bar graphs showing quantification of BrdU positive cells (mean  $\pm$  SEM) (A) 8 hours (B) 24 hours and (C) 72 hours post-treatment in C57BL/6 mice. \*,  $p < 0.01$  vs. vehicle (one way ANOVA followed by Bonferroni multiple comparison test;  $n = 4$  mice/group).

data indicate that exogenous and endogenous cannabinoids induce progenitor cell proliferation that is observed 24 hours after administration and is sustained up to 72 hours.

#### **Cannabinoids do not regulate differentiation in the olfactory epithelium**

The cannabinoid system contributes to the regulation of central nervous system neural precursors committed to a neuronal or glial lineage.<sup>18</sup> Therefore, to determine if cannabinoids induce the proliferation of neuronal and sustentacular progenitor cells, the BrdU incorporation assay was extended to 16 d following WIN or JZL/URB administration in C57BL/6 adult mice. This extended period allows for the basal cells to proliferate and differentiate into mature olfactory sensory neurons, a process that takes a minimum of 10 d.<sup>10</sup> To analyze the fate of cannabinoid-induced cell proliferation, BrdU positive cells in the total olfactory epithelium, the apical sustentacular cell layer, the middle neuronal layer, and the basal progenitor cell layer were counted. As demonstrated in Figure 3A, both WIN and JZL/URB significantly increased proliferation compared to vehicle at 2 d post-treatment in the total olfactory epithelium (Fig. 5A;  $53.5 \pm 2.2$  and  $68.1 \pm 5.0$  v.  $34.0 \pm 3.5$  cells/mm olfactory epithelium, respectively;  $p < 0.01$ ). There was no change in BrdU incorporation in vehicle treated mice between 2 and 16 d (Fig. 5A;  $34.0 \pm 3.5$  vs.  $34.6 \pm 3.2$  cells/mm olfactory epithelium;  $p > 0.05$ ). Additionally, at 16 d post-treatment there was no change in BrdU incorporation following treatment with WIN or JZL/URB compared to vehicle (Fig. 5A;  $37.2 \pm 1.0$  or  $33.7 \pm$

$2.8$  vs.  $34.6 \pm 3.2$  cells/mm olfactory epithelium, respectively;  $p > 0.05$ ). Finally, BrdU incorporation was significantly lower at 16 d compared to 2 d post-treatment following administration of WIN (Fig. 5A;  $37.2 \pm 1.0$  v.  $53.5 \pm 1.7$  cells/mm olfactory epithelium;  $p < 0.05$ ) or JZL/URB (Fig. 5A;  $33.7 \pm 2.8$  v.  $68.1 \pm 5.0$  cells/mm olfactory epithelium;  $p < 0.001$ ), suggesting that cannabinoid-induced newly generated cells do not survive to 16 d. Not surprisingly, in the basal cell layer WIN and JZL/URB treatment significantly increased the number of BrdU+ cells 2 d after intranasal administration by  $73 \pm 7\%$  and  $99 \pm 29\%$  of the vehicle, respectively (Fig. 5B;  $48.8 \pm 1.9$  and  $56.1 \pm 8.2$  vs.  $28.2 \pm 3.2$  cells/mm olfactory epithelium, respectively;  $p < 0.01$ ). At 16 d post-treatment (vehicle, WIN and JZL/URB) there was no significant change in the number of cells that incorporated BrdU in the basal cell layer between all treatment groups (Fig. 5B;  $16.9 \pm 2.0$ ,  $20.4 \pm 0.5$ , and  $19.8 \pm 2.4$  cells/mm olfactory epithelium, respectively;  $p > 0.05$ ) and also compared to the vehicle treatment at 2 d post-administration (Fig. 5B;  $p > 0.05$ ). In the neuronal layer, neither WIN nor JZL/URB treatment changed BrdU incorporation compared to vehicle at 2 or 16 d post-institution (Fig. 5C;  $p > 0.05$ ), indicating under homeostatic conditions cannabinoid treatment does not induce increased neurogenesis. Similarly, no change in proliferation was detected in the apical layer at either 2 or 16 d after WIN or JZL/URB administration (Fig. 5D;  $p > 0.05$ ), suggesting the generation of non-neuronal cells is not modulated by cannabinoids. Collectively, these data suggest that cannabinoid-induced proliferation does not lead to an increase in differentiated cells above control levels in the mature



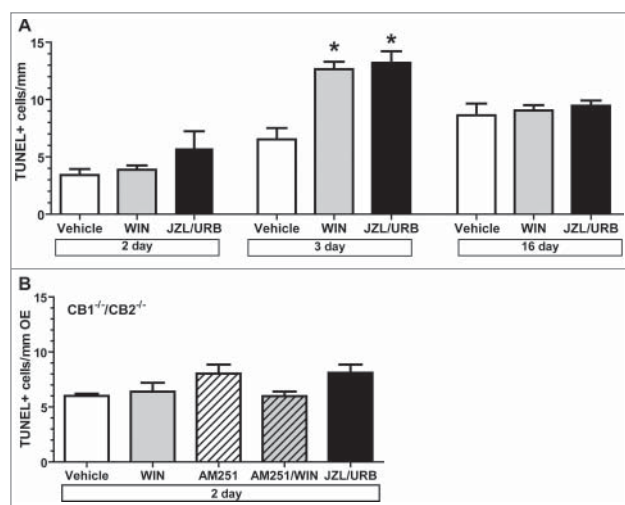
**Figure 5.** Cannabinoid administration does not regulate differentiation. Adult C57BL/6 mice intranasally aspirated vehicle (1% DMSO), WIN (10  $\mu$ M), or JZL/URB (10  $\mu$ M, 100  $\mu$ M, respectively). BrdU was injected (i.p., 18 mg/kg) at 42 and 45 hours and tissue was collected 2 or 16 d post-administration. Bar graphs showing quantitative analysis of BrdU incorporation in (A) the total olfactory epithelium, (B) the basal layer, (C) the neuronal layer, or (D) the apical layer. Data are expressed as mean number ( $\pm$  SEM) of BrdU positive cells per linear length of olfactory epithelium. \*,  $p < 0.01$  vs. vehicle at same time point, #,  $p < 0.05$  vs. same treatment at 2 d (one way ANOVA followed by Bonferroni multiple comparison test;  $n = 4$  mice/group).

olfactory epithelium. Although, the newly proliferated cells do not incorporate into the olfactory epithelium as mature neurons and glia, tightly regulated homeostatic mechanisms in the olfactory epithelium imply that cell death is occurring before maturation to maintain homeostasis.

### **Incidence of cell death after cannabinoid administration**

Given that cannabinoids induced an increase in proliferation but not an increase in newborn neurons, cell death was assessed at multiple time points after WIN or JZL/URB administration using TUNEL analysis. The mean number of TUNEL+ cells 2 d after administration of vehicle to C57BL/6 mice was  $3.4 \pm 0.4$  cells/mm tissue. Although the cell numbers were too low to quantify across regions of the epithelium, the TUNEL+ cells were observed primarily in the basal and middle neuronal layers of the epithelium. There was a significant effect of both cannabinoid treatment and time post-treatment on apoptosis in male C57BL/6 mice ( $F_{(2,34)} = 5.95$  and  $23.13$ , respectively;  $p < 0.01$ ). At 2 d post-treatment there was no significant difference between vehicle, WIN, and JZL/URB treatment (Fig. 6A,  $p > 0.05$  Neuman-Keuls

post-hoc analysis). However, apoptosis was significantly increased 3 d after WIN or JZL/URB treatment by  $49 \pm 9\%$  and  $51 \pm 15\%$  compared to vehicle (Fig. 6A;  $p < 0.01$ ;  $n=3$ ). At 16 d after WIN or JZL/URB treatment, the number of cells undergoing apoptosis was not significantly different compared to the respective vehicle (Fig. 6A;  $p > 0.05$ ). We also assessed whether pharmacological inhibition or genetic deletion of both CB1 and CB2 receptors has a protective function in the olfactory epithelium. There was no significant difference in TUNEL+ cells between vehicle, AM251, WIN, or JZL/URB treated CB1<sup>-/-</sup>/CB2<sup>-/-</sup> mice 2 d post-treatment (Fig. 6B;  $p > 0.05$ ). AM251 also had no effect on apoptosis in C57BL/6 mice 2 d post-treatment compared to vehicle ( $4.5 \pm 0.4$  v.  $3.4 \pm 0.5$  TUNEL+ cells/mm, respectively,  $p > 0.05$ ). Interestingly, there was a significant increase in TUNEL+ cells in vehicle treated CB1<sup>-/-</sup>/CB2<sup>-/-</sup> mice compared to C57BL/6 mice 2 d post-treatment ( $6.0 \pm 0.2$  vs.  $3.4 \pm 0.5$  TUNEL+ cells/mm;  $p < 0.01$ ). This increase in apoptosis compares to the increase in proliferation in the CB1<sup>-/-</sup>/CB2<sup>-/-</sup> mice. Overall, these data indicate that the cannabinoid-induced increase in proliferation is matched with an increase in cell death starting at 72 hours and ceasing within 2 weeks after administration.



**Figure 6.** Incidence of cell death following cannabinoid administration. Adult (6–8 week) male C57BL/6 or CB1<sup>-/-</sup>/CB2<sup>-/-</sup> mice (non-littermates) intranasally aspirated vehicle (1% DMSO), WIN (10  $\mu$ M), AM251 (10  $\mu$ M), AM251 prior to WIN, or JZL/URB (10  $\mu$ M, 100  $\mu$ M, respectively). Bar graphs showing quantification of TUNEL positive cells (mean  $\pm$  SEM) 2, 3 or 16 d post-treatment in C57BL/6 mice (A) or 2 d post-treatment in CB1<sup>-/-</sup>/CB2<sup>-/-</sup> mice (B). \*,  $p < 0.01$  vs. respective time vehicle (Two-way ANOVA followed by Newman-Keuls post-hoc test;  $n = 4$  mice/group).

## Discussion

We investigated the effect of cannabinoids on neurogenesis in the mouse olfactory epithelium *in vivo*. Neurogenesis is a finely tuned process involving the proliferation of progenitor cells, successful differentiation, and functional integration into the surrounding tissue. Cannabinoid administration increased proliferation as measured by BrdU-incorporation 48 hours following treatment in neonates during the late development stage (postnatal days 0–4) and in adults (6–8 weeks). Neonatal mice normally exhibit higher levels of proliferation compared to adult mice and cannabinoids are well known to regulate development during early post-natal periods. Not surprisingly, neonates expressed an inherently greater amount of proliferation compared to adults in the vehicle treatment groups. Of note is that WIN significantly increased BrdU incorporation by  $35 \pm 7\%$  compared to vehicle in the neonates and  $27 \pm 1\%$  above vehicle in the adults, suggesting that similar mechanisms and chemical signals may mediate both post-natal and adult neurogenesis under normal conditions.

BrdU-incorporation was increased with both exogenous cannabinoid WIN and by increasing endogenous cannabinoids by blocking their principal

hydrolytic enzymes (JZL/URB treatment) in control C57BL/6 mice. In the transgenic CB1<sup>-/-</sup>/CB2<sup>-/-</sup> mice, no difference in BrdU incorporation was seen in any treatment group. These data suggest cannabinoid-induced increases in proliferation are mediated by classical CB receptors or other receptors known to be activated by cannabinoids (e.g., TRPV1, peroxisome proliferator-activated receptors, orphan G protein coupled receptors) and not at off target receptors. WIN-induced proliferation was comparable in the adult outbred Swiss Webster mouse strain and the inbred C57BL/6 mouse strain. However, the inherent amount of proliferation that occurred in the vehicle-treated groups varied between strains indicating that there are strain differences in basal cell proliferation in the olfactory epithelium. The genetic variability with outbred strains coupled with the significant increase in proliferation suggests that the cannabinoid effect is robust. These data indicate that cannabinoids are a proliferative factor throughout development and into adulthood in the mouse olfactory epithelium.

Additionally, the CB1 receptor specific antagonist AM251 inhibited WIN-induced proliferation, further implicating CB1 receptors in this phenomenon. AM251 instillation alone significantly decreased the proliferation rate in adult Swiss Webster mice, suggesting that tonic release of cannabinoids can help regulate cell turn over. However, AM251 treatment did not decrease proliferation in the inbred C57BL/6 mouse strain. Tonic release of 2-AG is supported by the high steady-state levels of 2-AG found in the mouse olfactory epithelium.<sup>25</sup> High basal 2-AG levels are also detected in the rat brain,<sup>33</sup> and, coupled with multiple roles in lipid metabolism,<sup>34</sup> indicate that “reserve” 2-AG could function as a messenger for intercellular signaling.<sup>35–37</sup> Previous reports of 2-AG synthesis and release in the tadpole olfactory epithelium have suggested both autocrine and paracrine signaling routes,<sup>24</sup> therefore, similar mechanisms could be at work in the mouse olfactory epithelium. Future studies involving genetic depletion of 2-AG synthesis enzyme DAGL could further investigate the contribution of tonic cannabinoid stimulation on basal cell cycle regulation and proliferation. Cannabinoids may have a role in normal maintenance of basal cell proliferation state in the olfactory epithelium, in addition to other contributing factors.

A higher basal level of proliferation occurs in vehicle treated CB1<sup>-/-</sup>/CB2<sup>-/-</sup> mice compared to the C57BL/6 controls. Curiously, the increase in



proliferation persists despite decreased numbers of basal progenitor cells and olfactory sensory neurons in  $CB1^{-/-}/CB2^{-/-}$  mice.<sup>25</sup> This observation conflicts with previous reports of a robust physiological decrease in proliferation in both the dentate gyrus and subventricular zone in  $CB1^{-/-}$  mice,<sup>17</sup> and in the dentate gyrus of  $CB2^{-/-}$  mice.<sup>38</sup> To date, no studies have examined proliferation levels in the double knockout  $CB1^{-/-}/CB2^{-/-}$  mice. We recently discovered that  $CB1^{-/-}/CB2^{-/-}$  mice have a decreased population of mature olfactory sensory neurons.<sup>25</sup> Neurons in the olfactory epithelium negatively regulate neurogenesis via an inhibitory signal GDF11,<sup>39</sup> thus a decrease in the negative feedback signal could increase proliferation in the  $CB1^{-/-}/CB2^{-/-}$  mouse. The natural increase in BrdU incorporation in the  $CB1^{-/-}/CB2^{-/-}$  mouse is mirrored with overall increases in cell death.

A few mechanisms of CB-mediated proliferation have been identified in the central nervous system that may also play a role in the olfactory epithelium. (1)  $G\alpha_{i/o}$  protein coupled CB1 receptors promote proliferation through the phosphatidylinositol 3-kinase (PI3K)/AKT/glycogen synthase kinase (GSK)-3 $\beta$ / $\beta$ -catenin pathway in neuronal precursors from both the cerebellum and subventricular zone, suggesting that this pathway plays a pivotal role in the CB1 receptor-dependent modulation of neuronal proliferation.<sup>40,41</sup> CB1 receptor activation increases  $\beta$ -catenin nuclear localization which can then activate transcription factors that induce proliferation, thereby modulating cell cycle regulatory genes such as cyclin D1.<sup>40</sup> Recent evidence shows that  $\beta$ -catenin plays an important role in regulating proliferation of neural stem cells by decreasing cell cycle exit.<sup>42</sup> (2) Similarly, homeostatic factors can signal through the PI3K/Akt pathway to help control levels of proliferation and differentiation in the mouse olfactory epithelium. Neurotrophin-3, a neurotrophic factor, has been localized in a subpopulation of neurons within the olfactory epithelium,<sup>43</sup> and activates both the mitogen-activated protein kinase (MAPK) and PI3K/Akt pathways in vitro and inhibition of the PI3K/Akt pathway resulted in decreased survival of mature neurons.<sup>44</sup> (3) CB1 signaling in neural cells may involve the activation of mammalian target of rapamycin complex 1 (mTORC1), a protein kinase that regulates cell growth, proliferation, and survival.<sup>45</sup> CB1 receptor stimulation in hippocampal neurons activates mTORC1 and phosphorylation of downstream targets

that result in protein synthesis.<sup>46,47</sup> (4) Finally, direct endocannabinoid effects on neural progenitor differentiation may occur through extracellular signal-related kinase (ERK) signaling through a mechanism that involves the upstream inhibition of small GTPase Rap1 and proto-oncogene B-Raf.<sup>14</sup> MEK-ERK signaling is seen in the mouse olfactory epithelium in response to neurogenic signaling factor neuropeptide Y.<sup>48</sup> This indicates that CB receptor activation could also lead to proliferation via the MEK/ERK pathway, and that convergence of trophic factor signaling could occur between neuropeptide Y and endocannabinoids in the mouse olfactory epithelium.

Cell lineage experiments evaluated the location of BrdU incorporation in either the basal layer, neuronal layer (indicative of neurogenesis), or apical layer (indicative of non-neuronal cell generation) of the olfactory epithelium in adult C57BL/6 mice. No increases in newly proliferated cells were seen in any layer 16 d after WIN or JZL/URB treatment compared to vehicle control, suggesting that increases in proliferation did not lead to additional generation of cells. Additionally, a significant decrease in total BrdU cell numbers was seen 16 d after WIN and JZL/URB instillation compared to 2 days, suggesting the cannabinoid-induced increases in proliferation at 2 d are not maintained and newly proliferated cells undergo apoptosis before 14 d after treatment. These data further indicate that the cannabinoid system regulates the proliferative state of basal cells but not differentiation. We speculate that following an injury that required neurogenesis, cannabinoids may mediate the proliferation of neuronal progenitor cells that do differentiate into mature neurons. Note that a single instillation of cannabinoid agonist or JZL/URB was given while other studies examining the role of cannabinoids on differentiation involve multiple treatments.<sup>15,21</sup> During development, an inverse relation between expression of CB1 receptors and the stage of cell differentiation is seen in neuronal and glial cells,<sup>38</sup> indicating that CB1 receptors might function to prevent a differentiated state in favor of a non-differentiated, proliferative state. Similarly, CB2 receptors increase with de-differentiation of glial<sup>49</sup> and some tumors.<sup>50</sup> Collectively, these data suggest that cannabinoid signaling actively promotes basal cell proliferation while not influencing differentiation in C57BL/6 mice.

In the central nervous system, cannabinoids take part in the control of tissue homeostasis, thereby modulating the balance between apoptotic cell death, and cell protection and survival.<sup>51–53</sup> Both CB1 and CB2 receptors regulate neural progenitor commitment, survival, and cell-cycle maintenance.<sup>18,53</sup> WIN protects hippocampal neurons in culture from glutamate-induced excitotoxicity through a CB1 receptor-dependent mechanism.<sup>54</sup> Genetic deletion of CB1 receptors renders mice more sensitive to the harmful effects of ischemia, excitotoxic effects of glutamate, and oxidative injury.<sup>55</sup> However, in the present study CB1 receptor antagonist AM251 did not alter apoptosis 48 hours following administration. This suggests that potential tonic release of cannabinoids does not function to prevent cell death. However, there was a significant increase in apoptosis in CB1<sup>-/-</sup>/CB2<sup>-/-</sup> mice, suggesting that there is an increase in basal levels of apoptosis in the absence of CB receptor signaling. Conversely, administration of cannabinoids can decrease cell survival of various tumor cells in culture.<sup>53</sup> In the present study, a cannabinoid-induced increase in proliferation was matched by a ~50% increase in apoptosis 72 hours after WIN or JZL/URB administration. However, despite a significant increase in cell death, the number of cannabinoid-induced proliferating cells is 4 to 5 times higher than the increased numbers of TUNEL positive cells. This suggests that apoptosis of newly generated cells takes place over an extended period of time or that apoptosis peaks after 3 d. Cannabinoid-induced increases in proliferation is still seen 3 d after administration, falling within the previously characterized duration of basal cell proliferation, so it is not surprising that cell death numbers at 72 hours are not sufficient to restore balance between proliferation and cell death numbers. Additionally, the number of TUNEL positive cells significantly decreased 16 d post-cannabinoid instillation compared to 8 and 72 hours, suggesting that the range of apoptosis falls between 3 and 16 d. We did not quantify the number of apoptotic cells in each layer, however, qualitatively, apoptosis occurred in the basal and neuronal layers where the newly generated cells would be located. This suggests that the newly generated cells that are induced by cannabinoid signaling die before maturation in the adult. This is in contrast to the developing cortex, where cannabinoid signaling regulates the transition of progenitor cells from the apical to basal regions important in balancing self-renewal

and neurogenesis.<sup>56</sup> We hypothesize that in the adult olfactory epithelium, in the absence of a need for neuronal expansion or regeneration, homeostatic mechanisms overcome the increase in cannabinoid-mediated proliferation and regulate cell survival. Taken together, these data demonstrate that CB receptors clearly influence cell homeostasis involved in cell survival and death in the olfactory epithelium.

## Conclusions

These data indicate that CB receptor signaling promotes proliferation rather than differentiation in adult mouse olfactory epithelium. Identification of signaling molecules that influence progenitor cell proliferation will allow investigation of the mechanisms responsible for initiating enhanced neuroregeneration under aging and pathological conditions. This study advances our understanding of stem cell lineage and the trophic factor/signaling mediators responsible for progenitor cell proliferation, differentiation, and survival in the mouse olfactory epithelium. Trophic factor requirements of olfactory epithelium progenitor cells are similar to progenitor cells in the CNS<sup>12</sup> and cannabinoid signaling can now be added to this list.

## Materials and methods

### Animals

Adult male (6–8 weeks old) Swiss Webster mice and neonatal (postnatal day 0–4) and adult male C57BL/6 mice were obtained from Charles River Laboratories. Adult male CB1<sup>-/-</sup>/CB2<sup>-/-</sup> mice were used in some experiments as a negative control and were kindly provided by Dr. Norbert Kaminski (Michigan State University, MI) who obtained them from Dr. Andreas Zimmer (University of Bonn, Germany).<sup>57</sup> Homozygous CB1<sup>-/-</sup>/CB2<sup>-/-</sup> mice were bred with homozygous CB1<sup>-/-</sup>/CB2<sup>-/-</sup> mice and thus, use of wild-type littermates, which would minimize the impact of genetic background variability, was not possible. The CB1<sup>-/-</sup>/CB2<sup>-/-</sup> mice were backcrossed on the C57BL/6 strain, and therefore C57BL/6 mice were used as control mice. To minimize the possible effect of different animal husbandry conditions, C57BL/6 mice were maintained in the same animal facility as the CB1<sup>-/-</sup>/CB2<sup>-/-</sup> mice for 2 weeks prior to use. Mice were given food and water *ad libitum*. Animal rooms were kept at 21–24°C and 40–60% relative

humidity with a 12-h light/dark cycle. All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals as approved by Michigan State University Institutional Animal Care and Use Committee.

### ***In vivo apoptosis, proliferation, and lineage studies***

Test compounds were administered dropwise via a pipette tip placed immediately above the nares of a mouse held in a supine position, allowing the test compounds to be intranasally aspirated. Neonatal mice aspirated 10  $\mu$ l and anesthetized (4% isoflurane) adult mice aspirated 50  $\mu$ l of test compounds. Test compounds were obtained from Cayman Chemical Company and prepared as concentrated stock solutions in solvents and diluted with saline to the final working concentration as follows: synthetic CB1 and CB2 receptor agonist (+)-WIN 55212,2 (WIN; 10  $\mu$ M in 1% DMSO), endogenous cannabinoid analog 2-AG ether (1  $\mu$ M, 5  $\mu$ M, 10  $\mu$ M in 50% ethanol), CB1 receptor antagonist AM251 (10  $\mu$ M in 1% DMSO), and a mixture of endogenous cannabinoid hydrolytic enzyme inhibitors JZL184 and URB597 (JZL/URB; 10  $\mu$ M and 100  $\mu$ M, respectively in 1% DMSO). The concentrations were  $\sim$ 100 times greater than reported  $K_d$  or  $K_i$  values to account for possible drug expulsion through the nares with intranasal administration and the need to dissolve through mucous to reach the epithelium. Vehicle control solutions used were 50% ethanol, or 1% DMSO, as appropriate. In the olfactory epithelium, the vehicle treatments used in this study induced similar proliferation as saline.<sup>58</sup> To examine CB1-receptor specific effects, a subset of mice intranasally aspirated CB1 receptor antagonist AM251 (10  $\mu$ M) or vehicle (1% DMSO) 30 minutes prior to test compounds.

In order to detect proliferation through BrdU-incorporation, animals received 2 BrdU injections (i. p., 18 mg/kg total) at 2 and 4 hours (neonates) or 6 and 3 hours (adults) prior to tissue collection at 48 hours post-aspiration. In the neurogenesis lineage study, olfactory epithelium tissue was collected at 8 hours, 24 hours, 48 hours, 72 hours, or 16 d post-test compound administration. Tissue was processed and collected as described previously.<sup>29,59</sup> Tissue was always compared from equivalent levels in the

olfactory epithelium between treatment groups (n = 3–6 animals per group).

Apoptosis was examined using Terminal dUTP Nick End Labeling (TUNEL) performed with an In Situ Cell Death Detection Kit (TMR Red #12156792910; Roche Applied Science) following the manufacturer's instructions. BrdU immunoreactivity was assessed as described previously<sup>59</sup> using rat anti-BrdU immunoglobulin (1:100; ab6326, Abcam Inc.). For cell lineage studies, goat anti-olfactory marker protein immunoglobulin (OMP, 1:1000, Waco Chemicals) was co-applied with the rat anti-BrdU immunoglobulin. Immunoreactivity was detected using fluorescein isothiocyanate-conjugated donkey anti-rat immunoglobulin  $\pm$  Cy3-conjugated donkey anti-goat immunoglobulin (Jackson ImmunoResearch; 1:200 in 0.3% triton X 100 + 10% donkey serum in phosphate buffered saline for 2 hours at 37 $^{\circ}$  C). Sections were mounted with Vectashield mounting media for fluorescence (Vector Laboratories) and in some instances, nuclei were counterstained with Vectashield mounting medium for fluorescence with 4',6-diamidino-2-phenylindole (Vector Laboratories). Antibody specificity was examined by omitting the primary antibody; no immunoreactivity was ever observed. Immunoreactivity was visualized using a Nikon Eclipse 2000-U microscope equipped with an Excite 120 Fluorescence Illumination system or an Olympus FV1000 confocal laser scanning microscope. Fluorescent dyes were excited at 488 and 543 nm and low pass filtered at 505–525 and 560–620 nm, respectively. In some instances, the brightness and contrast of the fluorescent images were altered *post-hoc*. In all cases, the same changes were applied to all images collected on that given day, and it was verified that immunoreactivity was not observed in the antibody specificity controls under the new settings. No immunoreactivity was observed in any of the controls.

### ***Data analysis***

The number of BrdU positive or TUNEL positive cells in ectoturbinate 2 and endoturbinate II from 3 tissue sections of each mouse (n = 3–6 mice per group) were manually counted using 40X magnification by an experimenter blinded to the treatments. The ectoturbinate 2 and endoturbinate II are entirely comprised of olfactory epithelium and the linear length of these turbinates was determined by tracing the basement

membrane using MetaMorph software (Molecular Devices). Data collected from neonates were variable across experiments and so to enable comparison are expressed as normalized data (% of vehicle). We speculate that the difference in age of the neonates used, ranging from 0 day to 4 days, may be the cause for the variability given that the post-natal period is a time of tremendous expansion of the olfactory epithelium with high proliferation rates. Data collected from adults are expressed as a ratio of BrdU- or TUNEL-positive cells to the linear length of olfactory epithelium scored. In some experiments, BrdU positive cells were counted in (1) the apical sustentacular cell layer, defined as the region where the apical-most cell nuclei reside above OMP immunoreactivity, (2) the middle neuronal layer, defined as the region with OMP immunoreactivity, and (3) the basal cell layer, defined as the region below OMP immunoreactivity. The entire breadth of all 3 layers is designated "Total." This was performed to distinguish between basal cell, neuronal, and sustentacular cell proliferation, and to examine differentiation qualitatively. TUNEL-positive cells were quantified in the "Total" olfactory epithelium rather than in the different cell layers due to the low number of TUNEL+ cells observed. Student's t-test, and one-way ANOVA followed by the Bonferroni multiple comparison test were performed using Prism 5 (Graphpad Software). If there was significant variance as determined by Bartlett's test for equal variance, then the data were converted to their reciprocals to equalize the standard deviations. Two-way ANOVA or repeated-measures 2 ANOVA were performed followed by the Newman-Keul post hoc test using GB-Stat v9.0 (Dynamic Microsystems, Inc.).

### Abbreviations

2-AG	2-arachidonyl glycerol
BrdU	5'-bromodeoxyuridine
CB1	cannabinoid type 1 receptor
CB2	cannabinoid type 2 receptor
CB1 <sup>-/-</sup> /CB2 <sup>-/-</sup>	CB1 and CB2 deficient
JZL/URB	JZL184 and URB597
OMP	olfactory marker protein
TUNEL	Terminal dUTP Nick End Labeling
WIN	WIN 55212-2

### Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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