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Rapid ketamine effects on adult-born neurons

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Abstract

Virtually all antidepressants increase the birth of granule neurons in the adult dentate gyrus in rodents, providing a key basis for the neurogenesis hypothesis of antidepressant action. The novel antidepressant ketamine, however, shows antidepressant activity in humans within hours – far too rapid for a mechanism involving neuronal birth. Ketamine could potentially act more rapidly by enhancing maturation of new neurons born weeks earlier. To test this possibility, we assessed the effects of S-ketamine injection on maturation, as well as birth and survival, of new dentate gyrus granule neurons in rats, using the immediate-early gene zif268, PCNA, and BrdU respectively. We show that S-ketamine has rapid effects on new neurons, increasing the proportion of functionally-mature young granule neurons within 2 hours. A single injection of S-ketamine also increased cell proliferation and functional maturation, and decreased depressive-like behavior, for at least four weeks in rats treated with chronic corticosterone (a depression model) and controls. However, the behavioral effects of S-ketamine on neophagia were unaffected by elimination of adult neurogenesis. Together, these results indicate that ketamine has surprisingly rapid and long-lasting effects on recruitment of young neurons into hippocampal networks, but that ketamine has antidepressant-like effects that are independent of adult neurogenesis.

Significance Statement

Ketamine is a novel antidepressant that works very rapidly. Although ketamine acts as an antagonist of NMDA-type glutamate receptors, it is not clear how or where in the brain it acts to produce its antidepressant effects. This study demonstrates that ketamine has very rapid effects on the maturation of hippocampal neurons born in the adult brain, which have been linked to depression. However, behavioral experiments showed antidepressant-like effects of ketamine on neophagia that are independent of new neurons, in contrast to the effects of classical antidepressants on this behavior. Ketamine effects on new neurons should be considered as potential side effects of treatment and also point to a role for NMDA receptors in the normal maturation of new neurons.

Introduction

Major depression is a complex and disabling psychiatric disorder that is commonly treated with monoaminergic agents, such as serotonin-selective or norepinephrine-selective reuptake inhibitors (SSRIs and NRIs, respectively). These standard antidepressants have, in addition to limited therapeutic efficacy, delayed onset of action, requiring several weeks of treatment to produce clinical improvement (Browne and Lucki, 2013; Abdallah et al., 2015; Iadarola et al., 2015). These limitations point to the need for more effective and faster-acting antidepressant treatments.

Beyond the monoaminergic system, accumulating evidence supports a role for glutamatergic transmission in depression. Abnormalities in glutamate levels in plasma and brain tissue, as well as alteration in glutamate (AMPA, kainate, NMDA) receptor function have been reported in depressed patients(Sanacora et al., 2008). Evidence suggests that adaptive changes in NMDA receptor expression and function may even represent a final common pathway for monoaminergic antidepressants (Skolnick et al., 1996; 2009). Supporting the idea that NMDA receptors represent promising antidepressant targets, several clinical studies have reported antidepressant response to a single low dose infusion of ketamine, a noncompetitive NMDA receptor antagonist (Abdallah et al., 2015; Iadarola et al., 2015). Remarkably, antidepressant activity is observed in depressed patients resistant to prior treatments, begins in less than 4 hours, and is relatively sustained, lasting at least 1-2 weeks (Abdallah et al., 2015; Iadarola et al., 2015). Antidepressant effects of ketamine at subanesthetic doses have also been reported in rodent models of antidepressant efficacy, such as the forced swim and tail suspension tests (Browne and Lucki, 2013). Preclinical studies suggest that ketamine may exert its antidepressant activity through alterations in AMPA receptors, BDNF, mTOR, GSK-3, and formation of new dendritic spines and synapses in the prefrontal cortex (Browne and Lucki, 2013; Duman, 2014). However, other evidence points to a role for the hippocampus in ketamine's antidepressant effects (Kavalali and Monteggia, 2015).

A key feature of the hippocampus is the ongoing production of granule neurons in the dentate gyrus throughout life. The now well known “neurogenesis hypothesis” of antidepressant action has linked this adult neurogenesis to mood disorders and their treatment (Duman et al., 2001; Warner-Schmidt and Duman, 2006). Six classes of monoaminergic antidepressants all increase proliferation of granule cell precursors (Duman, 2004). Importantly, the delayed onset of antidepressant action parallels the time course of changes in neurogenesis. Although SSRIs immediately change extracellular levels of serotonin, several weeks of SSRI treatment are required to improve clinical symptoms in humans or to increase neurogenesis in animals (Malberg et al., 2000; Warner-Schmidt and Duman, 2006). Even if antidepressants were to rapidly increase cell proliferation, the additional new neurons would likely not contribute to behavior for several weeks, after maturation and functional integration into the local hippocampal network (Piatti et al., 2006; Snyder et al., 2009a). This maturation delay suggests that the rapid behavioral effects of ketamine treatment are not due to the birth of new neurons.

Acceleration of neuronal maturation, a much later stage in the neurogenic process, may represent an important target for novel more rapidly acting antidepressants. Previous studies have found that some antidepressants accelerate maturation after chronic treatment. Agomelatine, a novel antidepressant with mixed MT1/MT2 melatonin receptor agonist and 5-HT_{2C} receptor antagonist properties, accelerates maturation of young granule cells after 8 days of treatment (Soumier et al., 2009). Chronic treatment with the SSRI fluoxetine also facilitates the functional maturation of newly generated immature neurons after 21-days, but not 5 days, of treatment, paralleling its effects on behavior (Wang et al., 2008). One of the fastest and most effective antidepressant treatments is electroconvulsive therapy (Warner-Schmidt and Duman, 2006); electroconvulsive seizures also stimulate dendritic outgrowth and maturation (Overstreet-Wadiche et al., 2006). Taken together, these studies suggest that acceleration of hippocampal granule cell maturation could produce antidepressant effects. Therefore, we hypothesized that ketamine might rapidly

enhance neuronal maturation to immediately increase the pool of functional young neurons in the hippocampus and that this increase could reduce depressive-like behaviors.

We assessed the rapid and sustained effects of S-ketamine, an enantiomer of ketamine with robust effects and possibly fewer side effects (Mathew et al., 2012), in rodent behavioral tests that normally require chronic antidepressant treatment. We then determined the acute effects of S-ketamine on the functional maturation of young neurons and the long-term effects of S-ketamine on granule cell birth, maturation, and survival. Finally we tested the role of new neurons in ketamine's antidepressant-like action on novelty-suppressed feeding.

Methods and Materials

Animals

For most experiments, adult (8-week-old) male Long-Evans rats were ordered from a vendor (Charles River) and pair housed under a standard 12-hour light cycle with free access to food and water for at least one week prior to the start of experiments. For testing the role of neurogenesis in novelty-suppressed feeding (NSF) behavior, rats expressing herpes simples virus – thymidine kinase (HSV-TK) under the GFAP promoter (GFAP-TK rats) were generated on a Long-Evans background, using a construct previously used to make transgenic mice (Snyder et al., 2011). Male offspring were genotyped by PCR after weaning, and wild type and transgenic littermates were randomly pair housed under a standard 12-hour light/dark cycle for the duration of the experiment. All animal procedures were performed in accordance with the [Author University] animal care committee's regulations.

Drug Treatments

Experiment 1: Behavioral effects of S-ketamine

1a: Novelty Suppressed Feeding

Rats were food deprived for 24h. S-ketamine (S-(+)-ketamine hydrochloride, Sigma), at one of three doses (2.5, 5, or 10 mg/kg, all 2 ml/kg in 0.9% saline, i.p.), or saline was injected one hour prior to testing. The highest dose used here, 10 mg/kg, is 7.5-10x lower than anesthetic doses in rats; racemic mixtures at this dose produce antidepressant-like effects without altering spontaneous locomotor activity (Hunt et al., 2006; Wilson et al., 2007; Garcia et al., 2008; Engin et al., 2009; Li et al., 2010). One hour later, animals were placed in a brightly illuminated opaque Plexiglas box (50 cm × 50 cm × 40 cm) with six pellets of regular chow in the center. Behavior was video recorded from above for 10 min, and latency to feed was determined from recordings.

1b: Forced Swim Test

Rats were individually placed in a 50 cm high clear cylinder containing water (23 ± 1°C, 30 cm depth) for 15 min. One day later, rats were placed in the water again for a 5 min test. Each rat received 3 injections of either S-ketamine (10 mg/kg, i.p., Sigma), fluoxetine (10 mg/kg, i.p., Sigma), or saline, 24h, 4h, and 30min prior to the test session (Porsolt et al., 2001). To assess long-term effects, rats were placed in water again for 5 minutes 21 days after drug injection. Swim sessions were video recorded from the side, and immobility, swimming, and climbing behaviors were scored at the end of each 5s period from the recordings (Cryan et al., 2005).

Experiment 2: Acute effects of S-ketamine

2a: Effects 16 hours after S-ketamine

Rats were injected with bromodeoxyuridine (BrdU, 200 mg/kg, i.p., 10 mg/ml in saline with 0.007N NaOH) to identify young granule cells for maturation and survival analyses. Beginning 2 days after BrdU injection (Day 2), rats were injected daily with saline for 14 days to match the daily injections used for chronic treatment in Experiment 3. On Day 16, rats were given a single injection of either S-ketamine (10 mg/kg, i.p., Sigma), or saline. Sixteen hours later (Day 17), they were injected with kainic acid (15 mg/kg, i.p., Tocris) to drive immediate-early

gene expression in synaptically integrated granule cells (Snyder et al., 2009a; 2009b). Sodium pentobarbital (50 mg/kg; i.p., Ovation Pharmaceuticals) was given to stop seizures 30 min after the onset of stage 5 seizure activity. Rats were perfused 90 min after the onset of stage 5 seizure onset.

2b: Effects of S-ketamine in 7-day-old cells

Rats were treated exactly as above in Experiment 2a, except that ketamine was injected on Day 7 after BrdU, and no saline injections were given on intervening days.

2c: Effects 2 hours after S-ketamine

Rats were treated exactly as above in Experiment 2a, except that ketamine was injected on Day 14 after BrdU, no saline injections were given on intervening days, and kainic acid was injected 2 hours after saline or ketamine (also Day 14).

Experiment 3: Effects of chronic S-ketamine

Rats were injected with BrdU as above. Beginning on Day 2 after BrdU injection, rats were injected daily with S-ketamine (10 mg/ml, i.p., Sigma) or saline. After 14 or 21 days of daily ketamine injection, rats were injected with kainic acid followed by sodium pentobarbital as above and were perfused 90 min after the onset of stage 5 seizure onset.

Experiment 4: Sustained effects of ketamine in a depression model

Experiment 4a: Sucrose preference test and survival effects

Rats were injected with BrdU as above. On Day 2 after BrdU injection, rats were injected once with either S-ketamine (10 mg/kg, i.p., Sigma) or saline and then given corticosterone in their drinking water (400 µg/ml in 2.5% ethanol/water, v/v, equivalent to 40 mg/kg/day; Sigma) to produce a depression-like state (Sterner and Kalynchuk, 2010). Control rats drank 2.5% ethanol/water. On Day 30, corticosterone and ethanol were removed from the drinking water, and rats were given sucrose solution (1% in drinking water; Sigma) in one bottle

in addition to normal drinking water for a 48-hour habituation. The location of the sucrose bottles (left/right) was balanced across animals and was alternated after 24h. After a 4-hour water deprivation at the beginning of dark phase, rats were given a 1-hour free choice test with two identical bottles, one filled with the sucrose solution and the other with water. Sucrose preference was calculated as the volume of sucrose solution over total fluid volume consumed. Rats were perfused on Day 32, just after sucrose preference testing.

Experiment 4b: Proliferation and maturation effects

Rats were treated as in Experiment 4a above, except that BrdU was injected 16 days after corticosterone was added to the drinking water, there was no behavior testing, and after 32 days of corticosterone treatment, rats were injected with kainic acid followed by sodium pentobarbital as above and were perfused 90 min after the onset of stage 5 seizure onset.

Experiment 5: Effects of S-ketamine on behavior in the absence of new neurons

Beginning at 8 weeks of age, GFAP-TK rats and wild type littermates were given valganciclovir to eliminate adult neurogenesis (100 mg/kg/week, p.o., for 2 weeks, then 20 mg/kg/week for 6 weeks). After 8 weeks of valganciclovir treatment, rats were tested for novelty-suppressed feeding behavior as above. Rats were food deprived for 24h prior to testing. One hour prior to testing, rats were injected with saline or with S-ketamine (10 mg/kg, i.p., gift from Irv Wainer at NIA). For testing, animals were placed in a brightly illuminated opaque Plexiglas box (50 cm × 50 cm × 40 cm) with six pellets of regular chow in the center. Behavior was video recorded from above for 10 min, and latency to feed was determined from recordings.

Histological procedures and analysis

Rats were perfused with 4% paraformaldehyde and brains sectioned at 40µm throughout the entire hippocampus. Complete series of sections were enzymatically immunostained for BrdU or PCNA or fluorescently immunostained for doublecortin (DCX) or BrdU and zif268 combined, using Alexa dye-conjugated secondary antibodies. Zif268 is a synaptic activity-dependent immediate-early gene that is a reliable marker of the maturity of adult-born neurons (Jones et al., 2001; Snyder et al., 2009a; 2009b; Jungenitz et al., 2013). BrdU+, PCNA+, and DCX+ cells in the granule cell layer and subgranular zone were counted stereologically, using a 40x objective, on 1:12 series of sections through the entire dentate gyrus. Cell counts were multiplied by 12 to estimate the total number in each rat. To quantify IEG expression, 25 BrdU+ cells per hemisphere from the dorsal dentate gyrus were analyzed for co-labeling of zif268 and NeuN+ using a 63x objective on a confocal microscope. Statistical analyses were performed using 2-way ANOVA with Bonferroni post hoc tests, one-way ANOVA followed by the Dunnett's post hoc test, or Student's *t* test as appropriate.

Results

Rapid and prolonged effects of ketamine on behavior

The short- and long-term behavioral effects of S-ketamine in rats were examined in three tests. The novelty suppressed feeding test (NSF), which is sensitive to chronic but not acute monoaminergic antidepressant treatment (Bodnoff et al., 1988), was used to assess the acute effects of ketamine at 3 different doses. Injection of 10 mg/kg ketamine 1h prior to testing significantly reduced the latency to feed in the novel environment by 47% (1-way ANOVA, $F_{(3,14)} = 6.61$, $P = 0.005$; *Holm-Sidak 10mg/ml vs saline, P = 0.004; Fig 1A), as previously observed with the racemic mixture (Li et al., 2010; Carrier and Kabbaj, 2013). Lower doses of 2.5 and 5 mg/kg had no effect, in contrast to what has been reported using 5 mg/kg of the racemic mixture (Carrier and Kabbaj, 2013).

The forced swim test (FST) is classically used to detect antidepressant activity in rodents following acute or short-term treatment (Porsolt et al., 2001).

Repeated FST, which can detect behavioral changes following chronic treatment with low doses of classical antidepressants (Cryan et al., 2005), was used to assess the sustained antidepressant effect of ketamine (Fig 1B). Short-term administration of ketamine (10 mg/kg, i.p., 24h, 4h and 30min prior to testing) significantly decreased immobility time by 30% in the first test and 20% 21 days later (main effect of treatment $F_{(2,9)} = 31.65$, $P = 0.0001$; main effect of time $F_{(1,9)} = 31.47$, $P = 0.0003$; treatment x time interaction $F_{(2,9)} = 0.002$, $P = 0.99$; ketamine vs saline $P = 0.0007$ 1st session and $P = 0.0006$ in 2nd session). Treatment with the typical SSRI fluoxetine, at a dose showing chronic but not acute effects in previous studies (Porsolt et al., 2001; Cryan et al., 2005), produced no effect in either session. These results indicate that low dose ketamine, unlike fluoxetine, produces antidepressant-like effects that begin within one day and last at least 3 weeks, extending the time course previously observed in mice (Maeng et al., 2008).

Ketamine rapidly accelerates functional maturation of new neurons in the dentate gyrus

Kainate induced strong expression of zif268 throughout the granule cell layer in both groups (Fig 2A). In control rats (Fig 3A), 32% of the 16-day-old neurons expressed zif268 in response to kainate activation, consistent with expectations for rat granule neurons at this time point (Snyder et al., 2009a). In ketamine-treated rats, the proportion of BrdU+/NeuN+ cells labeled with zif268 was 67% higher (t-test, $t_4 = 3.065$, $P = 0.0375$; Fig 3A), suggesting a rapid increase in synaptic integration of 2-week-old granule cells. The 50 min half-life of ketamine in the rat brain (Mathew et al., 2012) is short relative to the 16 hour post-ketamine delay in this experiment, arguing against any direct interaction between ketamine and kainate.

Increased granule cell precursor proliferation is a common feature of antidepressant treatments (Duman et al., 2001) and could play a role in long-term behavioral effects of ketamine. Mitotic cells were assayed 16 hours after ketamine injection using the endogenous marker PCNA (Fig 2B). Acute

administration of ketamine significantly increased the number of PCNA+ cells located in the subgranular zone by 25% (t-test, t_{10}= 2.42, *P* = 0.0359; Fig 3B), consistent with the increased cell proliferation produced by other NMDA receptor antagonists (Cameron et al., 1995; Nacher et al., 2001; Nacher and McEwen, 2006).

To test the maturation effects in younger cells, the experiment was repeated giving BrdU only 7 days prior to ketamine injection (Fig 3E). There was no effect of ketamine on zif268 expression in these 7-day-old cells (t-test, t_9= 0.98, *P* = 0.35; Fig 3D), supporting the specificity of kainate-induced IEG expression as a measure of synaptic integration and suggesting that new maturation can only be rapidly accelerated after they have reached a certain level of maturity.

Behavioral effects of ketamine have been observed within 2 hours of ketamine administration (Fig 1) (Garcia et al., 2008; Engin et al., 2009; Li et al., 2010), so functional maturation of 14-day-old granule cells was assessed at this very short time point. After 2 hrs, ketamine increased the proportion of 14-day-old cells expressing zif268 by 50% compared with saline (t-test, t_9= 2.33, *P* = 0.0450; Fig 3F). Ketamine also significantly increased the proportion of BrdU+ cells strongly immunoreactive for NeuN (t-test, t_9= 3.55, *P* = 0.0062; Fig 3G), another measure of neuronal maturity (Snyder et al., 2009a). Taken together, these data demonstrate that a low dose of ketamine very rapidly induces maturation of young granule cells.

Chronic daily ketamine treatment does not enhance single injection effects

Several studies have examined behavioral effects of chronic daily ketamine treatment (Browne and Lucki, 2013). To determine the effects of chronic treatment with ketamine on neurogenesis, animals received ketamine daily (10 mg/kg, i.p) for 15 or 22 days, with kainate injection and perfusion 16 hours after the last ketamine injection (Fig 4a,b). Chronic ketamine significantly increased the proportion of BrdU-NeuN-zif268+ cells compared to saline treated group at both time points (14 days: t-test, t_9= 3.20, *P* = 0.0108; 21 days: t-test,

t_{11}= 2.773, *P* = 0.0181; Fig 4C and 4D). These increases, however, were approximately half as large as those seen with acute treatment. PCNA cell counting showed no effect of chronic ketamine after 14 or 21 days (14 days: t_{10}= 0.035, *P* = 0.973; 21 days: t_9= 0.955, *P* = 0.365; Fig 4E and 4F).

The effects of ketamine on survival of new granule cells was examined after chronic treatment by counting BrdU-labeled cells. To isolate effects on survival from possible proliferation effects (Dayer et al., 2003), rats were given BrdU 2 days before ketamine treatment began. Chronic treatment with ketamine for 14 days had no effect on the number of surviving BrdU+ cells located in the granule cell layer (t-test, t_{10}= 1.03, *P* = 0.33; Fig 4G), consistent with the lack of effect of the NMDA receptor antagonist CPP on survival of young granule cells observed previously in mice (Tashiro et al., 2006). Interestingly, 21 days of ketamine treatment decreased new granule cell survival (t-test, t_{12}= 2.71, *P* = 0.0191; Fig 4H), producing the only negative effect of ketamine on neurogenesis observed in the study.

Prolonged effects in a depression model

To assess the duration of the effects of a single injection of ketamine, maturation and proliferation were examined 32 days after a single injection of ketamine (10 mg/kg, i.p) or saline. Ketamine effects were tested in control conditions and in a depressive-like state (Sterner and Kalynchuk, 2010) induced by chronic cort treatment (Fig 5C). Cort had no effect on zif268 expression, suggesting that maturation is unaffected by excess glucocorticoids (Fig 5A). Ketamine, however, significantly increased the proportion of 16-day-old cells expressing zif268 by 45% in both cort-treated and untreated rats (main effect of cort: $F_{(1,17)}$ = 0.00, *P* = 0.996; *main effect of ketamine: $F_{(1,17)}$ = 14.99, *P* = 0.0017; cort x ketamine interaction: $F_{(1,17)}$ = 0.0005, *P* = 0.982 by 2-way ANOVA), indicating that ketamine continues to accelerate neuronal maturation for several weeks, even in neurons born long after acute ketamine treatment.

A single ketamine injection increased cell proliferation 32 days later (main effect of ketamine: $F_{(1, 23)}$ = 7.44, *P* = 0.013; Fig 5B, C). This finding extends a

previous report that the NMDA receptor antagonist CGP43487 increases cell proliferation for at least 7 days (Nacher et al., 2001) but contrasts with a recent study showing no effect of ketamine on cell proliferation or DCX+ cells 29 days after ketamine injection (Brachman et al., 2015) – suggesting either a species differences or decreased efficacy of higher ketamine doses. Chronic exposure to corticosterone decreased the number of PCNA+ cells by 33% (main effect of cort: $F_{(1, 23)} = 8.35$, $P = 0.009$; Fig 5B), as expected based on previous studies (Wong and Herbert, 2005; David et al., 2009). Cort and ketamine had independent, additive effects that, when combined, resulted in a proliferation rate very close to the control level (cort x ketamine interaction: $F_{(1, 23)} = 0.00$, $P = 0.988$ by 2-way ANOVA; Fig 5B). A previous study found interactive effects suggesting that NMDA receptor activation acts downstream of corticosterone at acute time points (Cameron et al., 1998). The results observed here suggest that the sustained effects of ketamine, after the drug itself is out of the system, may not directly involve NMDA receptors.

To determine the effects of ketamine on anhedonia and survival, the experiment was repeated, giving BrdU prior to treatment and testing for sucrose preference (Fig. 5F), a model of anhedonia (David et al., 2009; Sterner and Kalynchuk, 2010). Rats treated with corticosterone for 28 days showed a 30% decrease in preference for sucrose compared to saline-vehicle treated animals (; Fig 5D, as previously observed (Gourley et al., 2009; Sterner and Kalynchuk, 2010). This effect was reversed by ketamine (10 mg/kg, i.p.) given 32 days before testing (1-way ANOVA, $F_{(2,15)} = 6.98$, $P = 0.0072$; $P = 0.0405$ versus saline/cort in post hoc test; Fig 5D), a change that is unlikely to have been produced by nonspecific changes in thirst or hunger, because body weights and the total volume consumed during the 1h test (≈30ml) showed no significant group differences ($P = 0.61$ and $P = 0.22$, respectively). This experiment demonstrates sustained antidepressant activity of a single dose of ketamine in a model of depression that requires chronic treatment for efficacy of classical antidepressants (Sterner and Kalynchuk, 2010). These results are consistent

with recently observed ketamine effects on NSF in a similar paradigm (Brachman et al., 2015).

No statistically significant effects of either cort or ketamine on BrdU+ cell survival were observed ($F_{(2,15)} = 1.12$, $P = 0.35$ by 1-way ANOVA; Fig 5E). The data suggest a possible inhibitory effect of corticosterone on survival but provide no hint of any effect of ketamine.

New neurons are not required for behavioral effects of ketamine on neophagia

To investigate whether rapid changes in maturation of young neurons are causally related to rapid antidepressant-like effects in rats, we tested the behavioral effects of S-ketamine on NSF in rats lacking adult neurogenesis (Fig 6A). This test was chosen because we and others have seen an antidepressant effect of ketamine in this test and because neurogenesis-dependent effects of fluoxetine have been found in this test (Santarelli et al., 2003; David et al., 2009). After 8 weeks of valganciclovir treatment, which virtually eliminated new neurons in the dentate gyrus of GFAP-TK rats (Fig 6B), NSF behavior was tested in GFAP-TK rats and wild type littermate controls. Injection of 10 mg/kg ketamine 1h prior to testing significantly reduced the latency to feed in the novel environment (main effect of treatment in 2-way ANOVA, $F_{(1,22)} = 13.47$, $P = 0.002$; Fig 6C), as seen in our initial experiment (Fig 1A). There was no main effect of genotype or treatment x genotype interaction, (main effect of genotype: $F_{(1,22)} = 0.09$, $P = 0.767$; treatment x genotype interaction: $F_{(1,22)} = 0.71$, $P = 0.411$ by 2-way ANOVA), indicating that new neurons are not required for the behavioral effects of S-ketamine in this test. Home cage consumption was not measured in this experiment, but previous studies in rats and mice have found no effect of acute ketamine on this measure (Autry et al., 2011; Li et al., 2011; Iijima et al., 2012; Gideons et al., 2014; Nosyreva et al., 2014). Neurogenesis-dependent effects on sucrose preference and forced swim behavior at baseline, in the absence of ketamine, prevented testing of the requirement for

neurogenesis in ketamine's antidepressant effects in these tests (Snyder et al., 2011).

Discussion

The present findings demonstrate that S-ketamine has both rapid and sustained effects on adult neurogenesis in the dentate gyrus. A single injection of ketamine increased functional maturation of young neurons within hours and continued to accelerate maturation for at least 4 weeks. An increase in cell proliferation was also observed shortly after ketamine treatment and was sustained for at least 4 weeks. Chronic daily treatment with ketamine had more limited effects and had a small but significant negative effect on new neuron survival. The rapid and prolonged cellular effects matched the time course of behavioral effects in the NSF test, FST, and sucrose preference test. However, direct testing of the relationship between neurogenesis and behavioral effects on neophagia showed that new neurons were not required for a ketamine-induced decrease in this depressive-like behavior.

Accelerated maturation of adult-born neurons has previously been observed, but only after treatments lasting one or more weeks. Treatment of mice with fluoxetine for 28 days increases newborn neuron dendritic length, while 5 days of treatment does not (Wang et al., 2008). Agomelatine, a melatonergic receptor agonist and 5-HT_{2C} receptor antagonist, accelerates NeuN expression in granule cells after 8 days (Soumier et al., 2009). A non-pharmacologic treatment, exercise, increases the proportion of mature young neurons after 21 days but not after 14 days (Snyder et al., 2009b). The current study shows that similar changes in maturation can be induced by ketamine within only 2 hours. Many studies have demonstrated formation of new synapses in the adult brain within days (Woolley and McEwen, 1992; Holtmaat and Svoboda, 2009), but ketamine's effects on circuit formation are surprisingly fast even by this standard. However, *in vitro* studies have demonstrated that dendritic spines on neocortical pyramidal

neurons can form de novo in response to glutamate within minutes (Kwon and Sabatini, 2011; Okabe, 2013).

Ketamine acts at NMDA receptors, which are found throughout the brain. Our experiments could not determine whether the key NMDA receptors are those on the new neurons themselves or whether the effect is indirect. Studies using specific antagonists suggest that ketamine's effects on synapse formation and depressive-like behavior are mediated through the NMDA receptors containing the NR2B subunit (Maeng et al., 2008; Li et al., 2010). NR2B-containing NMDA receptors, generally thought of as a developmental form of the NMDA receptor, are expressed on young granule cells and are required for a form of plasticity produced exclusively by young neurons (Snyder et al., 2001). Therefore, it is reasonable to suspect that the effects of ketamine on maturing granule cells occur directly through NR2B-containing NMDA receptors on these young neurons. Deletion of the NR1 subunit of NMDA receptors from young neurons decreases their survival in a cell-specific manner (Tashiro et al., 2006), supporting a direct role for NMDA receptors on the young neurons. The decrease in new neuron survival seen following 21-day treatment with ketamine is consistent with the genetic ablation effects, though the relatively small effects seen with ketamine treatment suggest that survival effects of transient blockade are small and may be offset by increased cell proliferation.

Because ketamine acts as an antagonist, blocking NMDA receptors, the current findings suggest that endogenous activation of NMDA receptors normally slows incorporation of new neurons into functional circuits. Neuronal activation is generally regarded as being an important positive modulator of neuronal maturation, so the enhancement of maturation by NMDA receptor blockade is somewhat counterintuitive. However, genetic ablation of NMDA receptors in developing CA1 pyramidal cells increases the number of functional synapses detected by slice physiology (Adesnik et al., 2008) – counter to expectations but consistent with our results. Developing neurons, including adult-born granule neurons (Chancey et al., 2013), have many silent synapses containing NMDA receptors but no AMPA receptors. According to the model developed by Adesnik

et al. (2008), low level stimulation of these NMDA receptors inhibits AMPA receptor trafficking to the post-synaptic density. Strong activation of NMDA receptors normally overcomes this inhibition at some point during development, but deletion of NMDA receptors, or perhaps in our case pharmacological blockade of the NMDA receptors, disinhibits AMPA receptor trafficking and results in greater numbers of AMPA receptor-containing, functional synapses. This AMPA receptor trafficking and stabilization of synapses occurs within minutes (Groc et al., 2006). This model is supported by findings that AMPA receptor activation is required for the antidepressant effects of ketamine (Maeng et al., 2008; Li et al., 2010; Autry et al., 2011; Koike et al., 2011). BDNF, which is upregulated by ketamine and has been suggested as a mediator of its antidepressant effects (Garcia et al., 2008; Duman, 2014), also increases CA1 pyramidal cell dendritic spine size, an indicator of synapse maturity, within 10 minutes (Hale et al., 2011).

The finding that ketamine decreases feeding latency in the novelty-suppressed feeding task in adult rats lacking adult neurogenesis indicates that new neurons are not required for the antidepressant-like effects of ketamine, at least in this task. No rodent model of depressive-like behavior is clearly predictive of efficacy or mechanism of action in humans (Nestler and Hyman, 2010; Fernando and Robbins, 2011; Hyman, 2012), so it is possible that neurogenesis could play a role in the antidepressant activity of ketamine in humans. However, NSF may be the best available test for assessing ketamine effects on depressive-like behavior in rodents for several reasons. First, behavioral changes in NSF faithfully model the time course of antidepressant effects in humans, requiring chronic treatment with SSRIs but only acute ketamine treatment. In addition, SSRI effects on NSF behavior, in contrast to those of ketamine, do require new neurons in mice (Santarelli et al., 2003), demonstrating a clear distinction from ketamine effects on this test. This difference suggests that ketamine and SSRIs act through a different mechanism, not just at the level of receptors and neurotransmitter systems but also at the level of neuronal populations. These findings further suggest that changes in adult neurogenesis

are not the final common pathway for all antidepressant effects on rodent behavior and may not be important for therapeutic effects of ketamine in humans. Ketamine effects on behavior can be blocked with drug infusions into the prefrontal cortex (Li et al., 2010), suggesting that this brain region does play a role in antidepressant effects. Changes in AMPA receptors, signaling molecules, and dendritic spines have been observed in the prefrontal cortex 24 hours after ketamine treatment (Browne and Lucki, 2013; Duman, 2014) but have not been described at earlier timepoints. Nonetheless, very rapid effects on synapse maturation, like those observed in the current study, may also occur in presumably mature neurons in the prefrontal cortex and could provide a mechanism for the behavioral effects. It may be difficult to capture these very rapid changes in mature neurons, where only a subset of spines is normally silent or otherwise immature and able to be modified.

Although ketamine does not appear to act through new neurons to produce its antidepressant effects in the NSF test, albeit with the caveats discussed above, its pro-neurogenic effects should be considered as a side effect of this therapeutic treatment. The sustained effects, including increased cell proliferation and maturation for at least 4 weeks after a single treatment, may be the most important effects to consider in this context. Whether this increase in new neurons and acceleration of their maturation would be expected to have positive or negative effects on mental health is not yet clear. Nor is it apparent whether effects on adult neurogenesis are important for ketamine's sustained behavioral effects, which was not directly tested in this study. However, the rapid effects of ketamine on new neurons may be valuable both for understanding the normal maturation of adult-born neurons and for studying synaptic effects of ketamine that may occur more globally including in neurons responsible for its antidepressant effects.

Financial Disclosures

The authors report no biomedical financial interests or potential conflicts of interest.

Figure Legends

Fig 1. Rapid and sustained behavioral effects of S-ketamine. ***A,*** Acute S-ketamine reduced latency to eat in the novelty suppressed feeding test (1-way ANOVA, $F_{(3,14)} = 6.61$, $P = 0.005$; *Holm-Sidak 10mg/ml vs saline, P = 0.004). ***B,*** In the repeated forced swim test, sub-acute administration of S-ketamine reduced the time spent immobile immediately and 21 days later, while fluoxetine had no effect (2 way-repeated measures ANOVA, treatment effect $F_{(2,9)} = 31.65$, $P = 0.0001$; time effect $F_{(1,9)} = 31.47$, $P = 0.0003$; treatment x time interaction $F_{(2,9)} = 0.002$, $P = 0.99$; *** $P < .001$ versus saline in post hoc test).

Fig 2. Examples of immunohistochemical markers. ***A,*** After kainate injection, all mature granule neurons and some BrdU-labeled (+) 16-day-old NeuN+ neurons expressed zif268, indicating synaptic activation. ***B,*** Dividing cells (arrows) were identified using PCNA immunohistochemistry. ***C,*** Cells surviving 2-3 weeks (arrows) were identified with BrdU immunohistochemistry (gray-brown); immunonegative cells were stained with blue-purple counterstain.

Fig 3. Rapid effects of ketamine on granule cell maturation and proliferation. ***A,*** S-ketamine increased the proportion of 16-day-old BrdU+ cells co-labeled with NeuN and zif268 (zif) 16 hours later (*t-test, $t_4 = 3.065$, $P = 0.0375$). ***B,*** S-ketamine increased the number of PCNA+ (dividing) cells in the subgranular zone 16 hours later (*t-test, $t_{10} = 2.42$, $P = 0.0359$). ***C,*** Animal treatment time course for acute effects; ketamine injection was 10 mg/kg, i.p. in each experiment. ***D,*** The maturation effect was not seen in 7-day-old cells (t-test, $t_9 = 0.98$, $P = 0.35$). ***E,*** Animal treatment time course for acute effects in young cells. ***F, G,*** Increased zif/NeuN co-expression and strong NeuN expression were seen in 14-day-old cells within 2 hours of ketamine treatment (zif: *t-test, $t_9 = 2.33$, $P = 0.0450$; strong NeuN: *t-test, $t_9 = 3.55$, $P = 0.0062$). ***H,*** Animal treatment time course for very rapid effects on maturation.

Fig 4. Effects of chronic ketamine treatment. ***A, B***, Animal treatment time courses; all ketamine injections were 10 mg/kg, i.p. ***C, D***, Chronic daily ketamine treatment for 14 (***C***) or 21 (***D***) days increased the proportion of zif/NeuN+ BrdU+ granule cells (*14 days: t-test, t_9= 3.20, *P* = 0.0108; *21 days: t-test, t_{11}= 2.773, *P* = 0.0181). ***E, F***, S-ketamine had no effect on cell proliferation when give daily for 14 days or 21 days (14 days: t_{10}= 0.035, *P* = 0.973; 21 days: t_9= 0.955, *P* = 0.365). ***G, H***, BrdU+ cell survival was unaffected by 14 d daily treatment with S-ketamine (t-test, t_{10}= 1.03, *P* = 0.33) but was decreased after 21 d (*t-test, t_{12}= 2.71, *P* = 0.0191). Values represent mean ± SEM (*n* = 6-7 per group).

Fig 5. Sustained effects of S-ketamine in a depression model. ***A***, Ketamine given 32 days earlier increased zif/NeuN expression in 16-day-old cells regardless of chronic corticosterone exposure (main effect of cort: $F_{(1,17)}$ = 0.00, *P* = 0.996; *main effect of ketamine: $F_{(1,17)}$ = 14.99, *P* = 0.0017; cort x ketamine interaction: $F_{(1,17)}$ = 0.0005, *P* = 0.9821 by 2-way ANOVA). ***B***, S-ketamine increased the number of PCNA+ cells 32 days later and reversed the inhibition of proliferation by chronic corticosterone (*main effect of ketamine: $F_{(1, 23)}$ = 7.44, *P* = 0.013; *main effect of cort: $F_{(1, 23)}$ = 8.35, *P* = 0.009; cort x ketamine interaction: $F_{(1, 23)}$ = 0.00, *P* = 0.988 by 2-way ANOVA). ***C***, Animal treatment time course for maturation and proliferation effects. ***D***, A single S-ketamine injection prior to chronic corticosterone (cort) prevented a decrease in sucrose preference (1-way ANOVA, $F_{(2,15)}$ = 6.98, *P* = 0.0072; **P* < 0.05 versus saline in post hoc test). Values represent mean ± SEM (*n* = 4-6 per group). ***E***, Neither chronic exposure to cort nor acute ketamine prior to cort significantly altered new cell survival ($F_{(2,15)}$ = 1.12, *P* = 0.35 by 1-way ANOVA). Values represent mean ± SEM (*n* = 6-7 per group. ***F***, Animal treatment time course for sucrose preference and survival effects.

626 **Fig 6.** Neurogenesis is not required for S-ketamine effect on novelty-suppressed
627 feeding. **A**, Animal treatment time course showing valganciclovir to inhibit
628 neurogenesis, injection of saline (sal) or ketamine (ket, 10 mg/kg), and novelty
629 suppressed feeding (NSF) testing. **B**, Photos show doublecortin (DCX)-
630 expressing young granule neurons (green) in the dentate gyrus of valganciclovir
631 (VGCV)-treated wild type rats but not GFAP-TK rats. Blue counterstain shows
632 cell nuclei. **C**, Higher magnification of granule cell layer showing DCX staining. **D,**
633 Quantification shows essentially complete absence of DCX+ new neurons in
634 GFAP-TK rats and no effect of acute S-ketamine on DCX+ cell number. **E,** In the
635 NSF test, the latency to eat in a novel arena was decreased by S-ketamine in
636 both wild type and GFAP-TK rats (*main effect of treatment in 2-way ANOVA,
637 $F_{(1,22)} = 13.47$, $P = 0.002$; main effect of genotype: $F_{(1,22)} = 0.09$, $P = 0.767$;
638 treatment x genotype interaction: $F_{(1,22)} = 0.71$, $P = 0.411$ by 2-way ANOVA).
639

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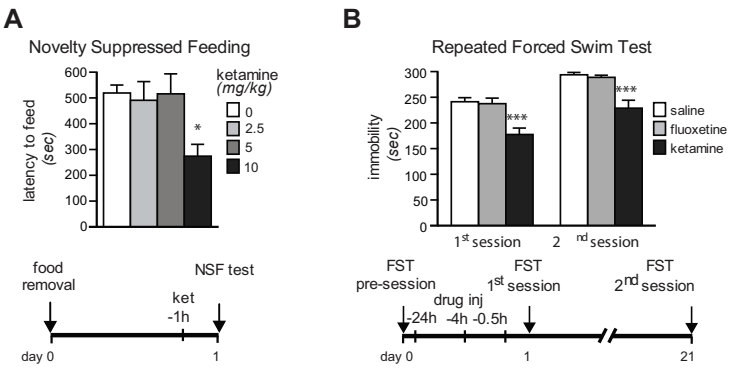

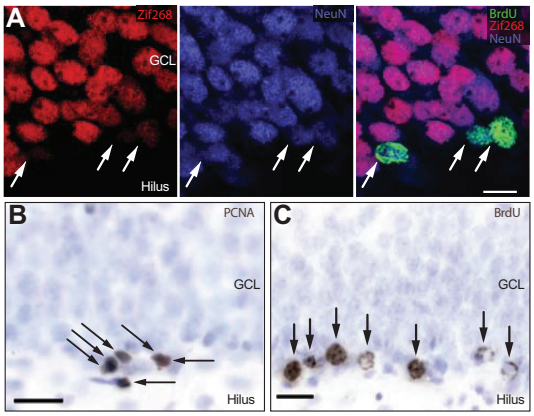

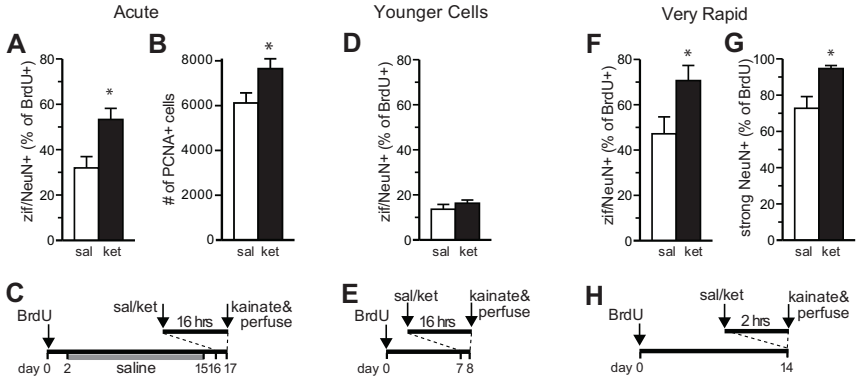

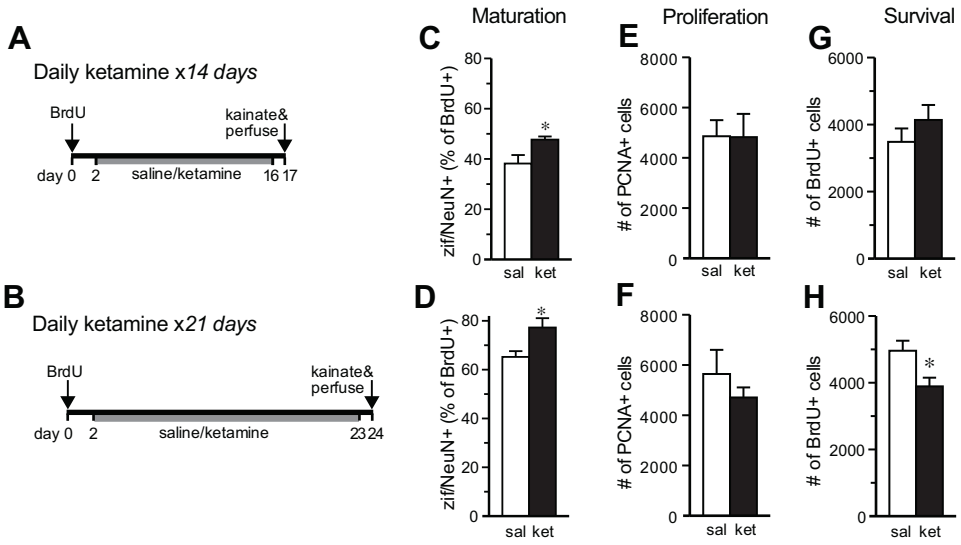

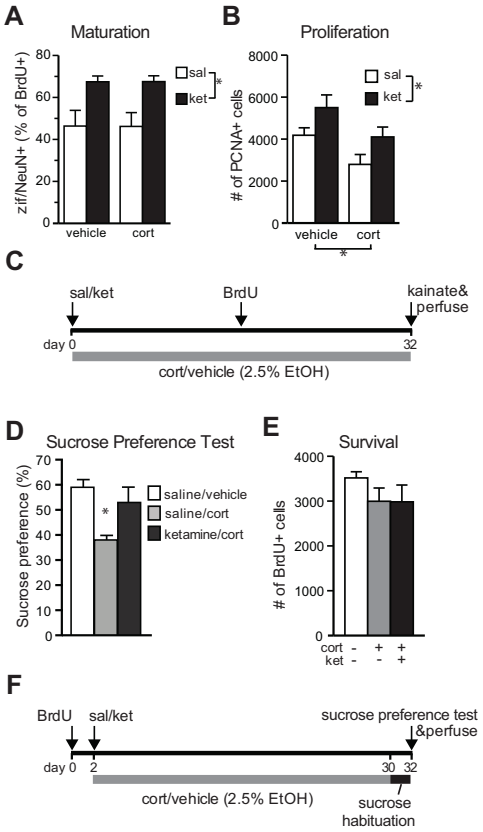

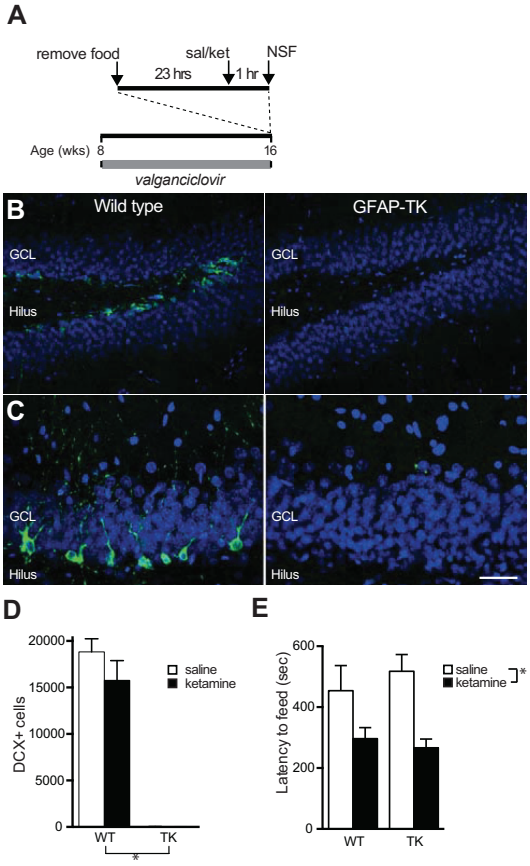

	Figure	Description	Data Structure	Type of test	Power
a	1A	latency to eat in NSF (2.5, 5, and 10 mg/kg ketamine vs saline)	Normal distribution	ANOVA	$p = 0.0052$
b	1A	latency to eat in NSF (10 mg/kg ketamine vs saline)	Normal distribution	Holm-Sidak post hoc test	$p = 0.0042$
c	1B	immobility in repeated FST, main effect of treatment	Normal distribution	2-way repeated measures ANOVA	$p < 0.0001$
d	1B	immobility in repeated FST, main effect of time	Normal distribution	2-way repeated measures ANOVA	$p = 0.0003$
e	1B	immobility in repeated FST, treatment x time interaction	Normal distribution	2-way repeated measures ANOVA	$p = 0.9980$
f	1B	immobility in repeated FST, ketamine vs saline post hoc	Normal distribution	Holm-Sidak post hoc test	$p < 0.0001$
g	3A	acute effects on cell maturation (ketamine vs saline)	Normal distribution	t test	$p = 0.0375$
h	3B	proliferation effects (ketamine vs saline)	Normal distribution	t test	$p = 0.0359$
i	3D	maturation effects (zif/NeuN expression) in younger cells (ketamine vs saline)	Normal distribution	t test	$p = 0.3548$
j	3F	very rapid maturation effects (zif/NeuN expression)	Normal distribution	t test	$p = 0.0450$
k	3G	maturation effects (strong NeuN expression) in 14-day-old cells (ketamine vs saline)	Normal distribution	t test	$p = 0.0062$
l	4C	chronic 14-day maturation effects (ketamine vs saline)	Normal distribution	t test	$p = 0.0108$
m	4D	chronic 21-day maturation effects (ketamine vs saline)	Normal distribution	t test	$p = 0.0181$
n	4E	chronic 14-day proliferation effects (ketamine vs saline)	Normal distribution	t test	$p = 0.9726$
o	4F	chronic 21-day proliferation effects (ketamine vs saline)	Normal distribution	t test	$p = 0.365$

p	4G	chronic 14-day survival effects (ketamine vs saline)	Normal distribution	t test	$p = 0.3280$
q	4H	chronic 21-day survival effects (ketamine vs saline)	Normal distribution	t test	$p = 0.0191$
r	5A	sustained maturation in CORT model, main effect of CORT	Normal distribution	2-way ANOVA (main effect)	$p = 0.9964$
s	5A	sustained maturation in CORT model, main effect of ketamine	Normal distribution	2-way ANOVA (main effect)	$p = 0.0017$
t	5A	sustained maturation in CORT model, CORT x ketamine interaction	Normal distribution	2-way ANOVA (interaction)	$p = 0.9821$
u	5B	proliferation in CORT model, main effect of ketamine	Normal distribution	2-way ANOVA (main effect)	$p = 0.0130$
v	5B	proliferation in CORT model, main effect of CORT	Normal distribution	2-way ANOVA (main effect)	$p = 0.0091$
w	5B	proliferation in CORT model, CORT x ketamine interaction	Normal distribution	2-way ANOVA (interaction)	$p = 0.9883$
x	5D	sucrose preference in CORT model	Normal distribution	ANOVA	$p = 0.0072$
y	5D	sucrose preference in CORT model, vehicle/saline vs. cort/saline	Normal distribution	ANOVA; post hoc test	$p = 0.0074$
z	5D	sucrose preference in CORT model, cort/saline vs. cort/ketamine	Normal distribution	ANOVA; post hoc test	$p = 0.0405$
aa	5E	survival in CORT model (ketamine vs saline)	Normal distribution	ANOVA	$p = 0.3512$