ABSTRACT

Rebecca Carter Feistritzer
The Effects of Stress-Induced Analgesia and Peripherally-Administered Cannabinoid Receptor Antagonists on Formalin-Induced Pain Behavior
Under the Direction of Dr. Andrea G. Hohmann

Stress-induced analgesia (SIA) occurs when naturally occurring brain constituents inhibit pain pathways upon exposure to a stressor and provide pain relief. These pathways involve cannabinoid receptors and their activation by their natural ligands, the endocannabinoids. The CB_1 antagonist AM251 and CB_2 antagonist SR144528 were used to block CB_1 and CB_2 receptors. The formalin test serves as a useful rodent model of pain in that an intradermal formalin injection inflicts two phases of pain behavior separated by a quiescent interval. Pain behaviors can be analyzed via quantification of time spent lifting, licking, or shaking the injected paw. Experiments were designed to compare the effects of SIA on formalin-induced pain and identify the role of peripherally-mediated cannabinoid receptors in contributing to endocannabinoid-mediated SIA in the formalin model. Rats were exposed to footshock to induce SIA before injections of varying concentrations of formalin. The composite pain score of pain behavior of shocked and non-shocked rats increased with increasing formalin concentrations, but was significantly lower overall in shocked rats. Thus, SIA effectively suppressed formalin-induced pain sensation. AM251 and SR144528 were injected intradermally in the paw before SIA induction and formalin injections. In animals subjected to footshock, SR144528 increased Phase 1 pain behavior compared to controls. By contrast, neither SR144528 nor AM251, administered locally in the paw. increased phase 2 behavior. In summary, SIA is effective at reducing formalin-induced pain, and blockade of cannabinoid CB_2 receptors in the paw attenuates SIA. Thus, peripherally-located CB_2 receptors play a role in stress-induced analgesia in the formalin test.

INDEX WORDS: Endocannabinoids, CB1, CB2, Stress-Induced Analgesia, Formalin, AM251, SR144528, DGLα, LacZ

The Effects of Stress-Induced Analgesia and Peripherally-Administered Cannabinoid Receptor Antagonists on Formalin-Induced Pain Behavior

by

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DEDICATION

To my parents,

Because of you, I am unlimited. You have shown me grace, and ‘tis grace that will lead me home.

ACKNOWLEDGEMENTS

I would first and foremost like to thank Dr. Andrea Hohmann. Without her patience and guidance I would not have been able to accomplish this project. Not many students get the chance to work with such a caring and devoted professor. I would also like to thank Dr. Jessica Spradley who taught me more than anybody else in the lab. She always helped and always explained. Finally, I wish to thank Dr. Richard Suplita. Without his previous research, mine would not have occurred.

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CHAPTER ONE
INTRODUCTION

Endocannabinoids, the brain's own cannabis-like compounds, mediate analgesia by binding to the cannabinoid receptors CB_1 and CB_2. The neural pathways involved in cannabinoid signaling as well as pain modulation include the periaqueductal grey matter (PAG) of the midbrain as well as the rostroventromedial medulla (RVM) of the brainstem [1].

Of the two receptor types, CB_1 mainly resides in the brain, and CB_2 is primarily found in immune tissues but is also present in the brain at low levels. Two endogenous cannabinoids have been identified that are ligands for CB_1 and CB_2 and are non-opioid based: arachidonylethanolamide (anandamide) and 2-arachidonoylglycerol (2-AG). These compounds are generated via stimulus-dependent cleavage of precursors found in membranes of neurons and immunocytes [1]. Specifically, the enzyme phospholipase C aids in the formation of 1,2-diacylglycerol upon which the enzyme diacylglycerol lipase (DGL) acts to form 2-AG.

The formalin test serves as an excellent model to examine the role of endocannabinoids in analgesia in rats. The noxious chemical formalin provides a continuous (rather than a transient) stimulus [2]. Formalin produces a measurable response to a long-lasting nociceptive stimulus with more central nervous system involvement [3]. This helps eliminate the problem of a pain threshold produced by other tests such as tail flick, pinch test, etc.[3]. The formalin test produces pain behavior that can be divided into two phases with the cut-off at 15 minutes. The early phase involves acute activation of pain-sensing C-fibers and peaks at five minutes before quickly

declining [4]. The second phase begins around 15 minutes when sensory fiber activity as well as inflammation and central sensitization cause sustained pain behavior [4]. An increase in formalin concentration causes an increase in nociceptive behavior. Previous studies have shown that in bicuculline-treated rats, this increase in pain behavior results from enhanced dorsal horn neuronal excitability and a decrease in inhibition in the spinal cord (instead of enhanced inflammation associated with increasing formalin concentrations) [5].

Formalin injections have been shown to increase the release of anandamide in the PAG, providing a framework in which to test the various involvements of anandamide in pain signaling [7]. However, the relationship between formalin and the endocannabinoid 2-AG have not been previously examined.

Stress-induced analgesia (SIA) occurs when neural systems release endogenous analgesic mediators to naturally inhibit pain following exposure to stress in the environment. Stress can cause a rapid accumulation of 2-AG in the midbrain, suggesting that endocannabinoid release mediates SIA instead of intrinsic CB_1 activity [6]. These non-opioid endogenous lipid mediators play a key role in this antinociception as demonstrated through the use of cannabinoid receptor antagonists [1]. Two cannabinoid receptor antagonists implemented in this study are AM251 and SR144528. AM251 drug targets the CB_1 receptor whereas SR144528 targets the CB_2 receptor.

When injected systemically, SR144528 increased pain behavior in the early, but not late, phase post formalin injection [4]. However, local injections of this antagonist were not evaluated in this study. Certain cannabinoid agonists (WIN-55212-2, HU-210) blocked both phases of pain behavior in mice models [4]. Additionally, exogenous anandamide was 100 times stronger

in preventing pain behavior when injected locally as opposed to intravenously [4]. Thus, based on previous research, cannabinoid receptor activation in the periphery plays a significant role in reducing formalin-induced pain. Local injections of cannabinoid antagonists are necessary to unveil the specific roles of each receptor subtype in suppressing formalin-induced pain.

In the present experiments, the formalin test was additionally paired with electrical shock to identify any possible relationship between SIA and formalin-induced pain suppression. Previous research has demonstrated SIA following stressful electrical foot shock via the tail-flick test [1]. A study of the effects of formalin concentration on pain behavior has been performed in mice, but no data exists regarding rats experiencing SIA [3].

Finally, RNA of the synthetic enzyme for 2-AG, DGL-α, was silenced via virally-mediated RNA silencing in the PAG, and its effects on antinociception were examined using the formalin test in the absence of SIA. If RNA silencing of DGL-α is supressed, then the formation of 2-AG should not occur.

Overall, we tested five related hypotheses: First, exposure to foot shock stress would produce SIA in the formalin test in rats. Second, increasing the concentration of formalin in the paw would increase pain behavior and attenuate SIA. Third, when using the antagonists, blockade of CB_2 receptors with SR144528 in the paw should block SIA and reinstate pain behavior in the formalin test. Fourth, phase 1 pain behavior should be more sensitive to CB_2-mediated SIA due to higher concentrations of CB_2 in the periphery. Fifth, injection of the DGL-α RNA silencing virus into the PAG should cause an increase in pain behavior post formalin injection due to the lack of 2-AG production.

CHAPTER TWO
MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats (n=84) weighing between 250-290 g were behaviorally tested. All rats were single-housed in plastic cages with wood shavings as bedding. Rats habituated to the animal facility for at least one week before testing, and access to food and water was provided *ad libitum*. The University of Georgia Animal Care and Use Committee approved all procedures.

Drug preparation

The CB_1 receptor antagonist AM251 (1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-(1-piperidyl)pyrazole-3-carboxamide) and the CB_2 antagonist SR144528 (N-[(1s)-endo-1,3,3-trimethylbicyclo[2.2.1]heptan-2-yl]5-(4-chloro-3-methylpanyl)-1-(4-methylbenzyl)pyrazole-3-carboxamide) were dissolved in 100% DMSO (1.6 μg/ 1μL). The vehicle was 100% DMSO:100% ethanol:100% cremophore:0.9% saline (1:1:1:2). The final concentration of the drug (80 μg) was administered subcutaneously (s.c.) in a volume of 50 μL.

Surgeries

Surgeries were performed to inject an RNA silencing virus in twelve of the animals anesthetized with Ketamine hydrochloride (100mg/mL) and Nembutal (50 mg/mL). The hair on the heads was shaved and the area cleaned. The stereotax was zeroed at bregma and lambda.

Holes were drilled at coordinates anterior-posterior (AP) 1.60 and medio-lateral (ML) 0.68 and -0.68. Saline cleared the needle each time before drawing up the virus and inserting it into the brain. The dura was ruptured and the needle was then lowered to dorsal-ventral (DV) 5.35 to accurately hit the PAG. Exactly 1.14 µL of either DGL-αi or a control virus LacZi were injected. This process was performed bilaterally for both ML coordinates. Bone wax was then placed in the holes created from drilling, and the scalp was stitched closed.

Study One: Formalin Concentrations

Rats habituated to the testing room for 15 minutes before being placed in a clear Plexiglass box that served as the observation chamber. The chamber rested on raised, clear glass with a mirror tilted at a 45° angle below so as to better observe the pain behavior. After habituating to the chamber for fifteen minutes, rats were placed in a shock box for 3 minutes. Half of the rats were shocked (3 mA) for three minutes while the other half were placed in the shock box for the equivalent time but received no shock. After being removed from the box, rats received a 50 µL injection of 0.09% formalin (n = 12), 0.5% formalin (n = 12), or 2.5% formalin (n = 12). As an additional control, six rats were injected with 2.5% formalin without being placed in the shock box at all. Observations were made every 5 minutes for 75 minutes to rank the different pain behaviors, as described below in section 3.7.

Study Two: CB_1 and CB_2 Antagonists

Rats habituated to the testing room for 15 minutes. Animals randomly received AM251 (n = 6; 80 µg in 50 µL), SR144528 (n = 6; 80 µg in 50 µL), or vehicle (n = 6; 50 µL) in the dorsal

surface of the right hind paw. The rats were then placed in the observation chamber (a clear Plexiglass box with mirror below) for an additional 15 minutes. Next, the animals were shocked (3 mA) for three minutes, and then received an injection of 2.5% formalin (50 μL) in the dorsal surface of the same right hind paw at a site neighboring the previous injection so as to avoid diluting the formalin concentration. Observations were made every 5 minutes for 75 minutes to rank the different pain behaviors. Additionally, the scorer was blind as to which of the three drugs had been administered to the animal, and the drugs were labeled either A (AM251), B (SR144528), or C (VEHICLE).

Study Three: Viral Vector

Two weeks prior to behavioral testing, twelve rats underwent surgeries described in the above section. The setup used for the viral vector testing allowed for the ability to test two rats at one time: two Plexiglass observation chambers sat on opposite ends of a rectangular piece of glass with a black box separating them to prevent the rats from seeing each other. The glass was raised with mirrors below as before. Two video cameras sat in front of each mirror, recording the animals' pain behaviors. After habituating to the testing room and the observation chamber for 15 minutes each, rats were injected with 50 μL of 2.5% formalin and were placed back into the chamber for 60 minutes. Scoring was done at a later date by a researcher blind to assigned treatments.

Pain Scoring

Nociception was recorded at three different levels. The first level is characterized by favoring of the injected foot where the rat does not put weight on it but still keeps it on the ground. Level two behavior involves the rat lifting the injected paw off of the ground. Level three behavior includes rapid lifts of the injected paw, licking, biting, or shaking. Weighted pain scores were calculated as follows:

$$\frac{1(\text{\# sec behavior two}) + 2(\text{\# sec behavior three})}{200 \text{ s}}$$

Data Analysis

The Area under the Curve of pain behavior during Phase 1 and 2 was calculated for each rat according to the trapezoidal rule. Additionally, pain behavior was compared between groups using ANOVA and planned comparison t-tests. Data was plotted as means ± the S.E.M. Each formalin concentration was analyzed between shock and no shock conditions. Each concentration was also compared to each of the other two concentrations within shock or no shock groups. The CB_1 and CB_2 antagonists were each compared to the vehicle as well as to each other. Finally, pain behavior was compared in animals injected with the virus engineered to silence DGLα mRNA against the LacZ control virus to determine any differences between groups. The significance level was set at $P < 0.05$.

CHAPTER THREE
RESULTS

Formalin Concentrations

Shocked rats overall demonstrated significantly less pain behavior compared to their non-shocked counterparts ($F_{6,34} = 34.92$, $p < 0.001$; $p < 0.01$ for each shock vs no shock/no box control condition). Planned comparisons across the first phase of the formalin test (0-15 minutes) demonstrated that 2.5% formalin produced significantly more pain behavior than the 0.5% formalin concentration (t=2.888, $p < 0.01$; Figure 1a, c), but no significant difference was observed between 2.5% formalin and 0.09% formalin concentrations. No significant differences were detected during Phase 1 across concentrations of formalin in animals receiving foot shock ($p = 0.2729$; Figure 1d). During Phase 2 (15-60 minutes) non-shocked animals showed differences in pain behavior depending on the concentration of formalin ($F_{2,13}= 11.81$, $p < 0.01$; Figure 1 e). Specifically, the animals receiving 2.5% formalin showed more pain behavior than either the 0.5% concentration or the 0.09% concentration ($p < 0.05$ for both post-hoc analyses). Furthermore across Phase 2, shocked animals had different pain scores depending on the concentration of formalin they received ($F_{2,16} = 12.49$, $p < 0.001$; Figure 1 f). Post-hoc tests revealed a significant difference between the 2.5% concentration and either of the two lower concentrations ($p < 0.05$ for both analyses).

During Phase 1, formalin pain was lower in shocked rats receiving the 0.09% concentration of formalin relative to non-shock animals receiving the same concentration of formalin (t_{10}=2.611, $p < 0.05$; Figure 2a-b). No change was observed in Phase 2 pain behavior between groups (Figure 2 c). The same relationship held true for the 0.5% formalin concentration across Phase 1 ($t_{10} = 4.012$, $p < 0.01$: Figure 3 a-c) in contrast to Phase 2. Foot shock specifically suppressed Phase 1 pain behavior in rats receiving 0.5% formalin without reliably altering pain behavior during Phase 2. Pain behaviors also differed depending on the shock box treatment the animals received ($F_{2,14} = 9.834$, $p < 0.01$; Figure 4 b). Specifically, the shocked rats showed less pain behavior than either non-shocked rats ($p < 0.01$) or rats that were not exposed to the shock box ($p < 0.05$). Likewise, during Phase 2 pain scores varied by shock box exposure ($F_{2,14} = 5.888$, $p < 0.05$; Figure 4 c). Post-hoc analyses revealed that shocked rats had attenuated pain scores relative to both the non-shocked ($p < 0.01$) and the non-exposed animals ($p < 0.05$). Additionally, the AUC of pain behavior was similar in no shock groups receiving 2.5% formalin that were placed in the chamber without shock (No shock group) or groups receiving the same concentration of formalin that were not exposed to the experimental chamber (No Box group) ($p = 0.46$).

CB_1 and CB_2 antagonists

Planned comparisons revealed that Phase 1 pain behavior was higher in animals receiving the CB_2 antagonist SR144528 compared to vehicle ($t_9 = 1.901$, $p < 0.05$) and approached significance versus the CB_1 antagonist AM251 ($t_9 = 1.74$, $p = 0.056$; Figure 5 a, b). Pain scores in animals receiving the CB_1 antagonist AM251 did not differ from vehicle ($p > 0.05$ for both

comparisons). However, during Phase 2 (15-75 min post-shock) no significant differences in pain scores were detected among the drug treatment groups ($p = 0.75$).

Viral Vector

No significant differences in pain behavior were found between the group receiving the DGL-α mRNA silencing viral vector and the group receiving the Lac Z control vector bilaterally in the dorsolateral PAG during either Phase 1 ($p = 0.22$) or Phase 2 ($p = 0.39$; Figure 6 a-c).

CHAPTER FOUR
DISCUSSION

Formalin Concentrations

Within each concentration, exposure to footshock stress significantly reduced pain behavior, and this can be attributed to SIA. At all concentrations of formalin, nociceptive behavior was eliminated during the Phase 1. During Phase 2, shocked animals mainly displayed pain behaviors of level two (data not shown), suggesting that rats engaged in lifting of the injected paw but not flinching, licking or biting. The increase in this type of behavior implies that inflammation and neuronal sensitization are the sources of pain as opposed to direct stimulation of the nerve endings by formalin [7]. Moreover, studies of 2-AG in platelets demonstrated the short life span of the compound, suggesting that degradation of 2-AG also factors into the elevation of pain behavior, and potentially the transient nature of peripheral endocannabinoid-mediated analgesia [1].

As predicted, pain behavior increased with escalating formalin concentration in shocked as well as non-shocked rats. The results of rats tested at 2.5% formalin without being placed in the box did not differ from the results obtained from rats placed in the shock box but not shocked. Thus, being in the shock box for 3 minutes did not have an effect on overall effect to dampen pain behavior. Additionally, the antinociception observed with shocked compared to non-shocked rats within a concentration did not arise from nerve damage at the foot from the

shock box. Rather, the difference results from SIA and is supported by the observation that nociception increased in shocked rats with increasing formalin concentrations. If nerve damage had occurred, increasing concentrations would not have increased pain behavior in shocked rats. Thus, SIA could be surmounted by increasing the concentration of formalin.

During Phase 1 (0-15 minutes pos-formalin), 0.09% formalin produced greater pain behavior than 0.5% formalin in non-shocked rats. However from 15-60 minutes (Phase 2), there was no difference in pain behavior between the two concentrations. In all cases, 2.5% formalin produced the greatest pain behavior in either rats exposed to foot shock or no shock. These results are consistent with a study of orofacial formalin tests: Clavelou et al [8] found that a Phase 2 response only strongly appeared with concentrations at or above 1.5% formalin.

CB_1 and CB_2 Antagonists

The two receptor antagonists did not produce a significant difference in the AUC of the two phases taken together (data not shown). However, SR144528 resulted in a partial return of pain behavior during the Phase 1 (0-15 minutes) when compared to the vehicle. Thus, SR144528 increased formalin-induced pain behavior and attenuated SIA. These findings support the idea that endocannabinoids are mobilized outside the CNS following exposure to a stressor. Additionally, our results suggest that endocannabinoids activate peripheral CB_2 receptors to produce stress-induced analgesia. The higher affinity of 2-AG, relative to anandamide, for CB_2 receptors also suggests that 2-AG mobilization during Phase 1 may account for SIA during that phase of the formalin test [9].

In previous studies demonstrating blockade of SIA by CB_1 antagonists, the drugs were injected systemically or directly into the brain. However, when injected in the periphery as done in this study, there was likely not enough time for the antagonist to reach the CNS before exposure to foot shock and injection of formalin. The lack of an increased overall behavior in animals that received pronociceptive drugs could be due to an increase in conflicting behaviors that prevent showing of pain such as freezing [8]. However, rats ambulated normally suggesting that freezing cannot account for the pattern of results obtained. Higher doses of antagonists or antagonist injection in multiple sites might be required to further attenuate SIA. These studies are also the first to show that endocannabinoid-mediated SIA has a peripheral component and can be detected in a model other than the spinally-mediated tail flick test.

Viral Vector

The DGL-α RNA-silencing viral vector did not increase pain behavior in rats that had been surgically injected beforehand with the virus. Preliminary research using a higher titer (which decreased 2-AG accumulation in the PAG) suppressed SIA following foot shock, and significantly increased formalin-induced pain (unpublished data: Spradley and Hohmann). However, the titer used during this experiment was likely too low to be effective at eliminating 2-AG production.

CHAPTER FIVE
CONCLUSION

Of the five hypotheses, the data presented here supported all except that motivating the DGL-α silencing experiment. Exposure to foot shock produced SIA in the formalin test. Furthermore, increasing the concentration of formalin can surmount the effects of SIA. The CB_2 receptor antagonist SR144528, administered locally in the paw, increased formalin-induced pain behavior, while the CB_1-receptor antagonist AM251 did not. Moreover, the pronociceptive effects of SR144528, administered locally to the paw, were observed specifically during Phase 1 of the formalin test, which is associated specifically with primary afferent activation. Finally, endocannabinoids were mobilized outside the CNS following exposure to a stressor, and they activate peripheral CB_2 receptors to suppress pain. Thus, these data emphasize the importance of peripheral cannabinoid receptors and SIA in reducing formalin-induced pain behaviors.

FIGURES

Figure 1

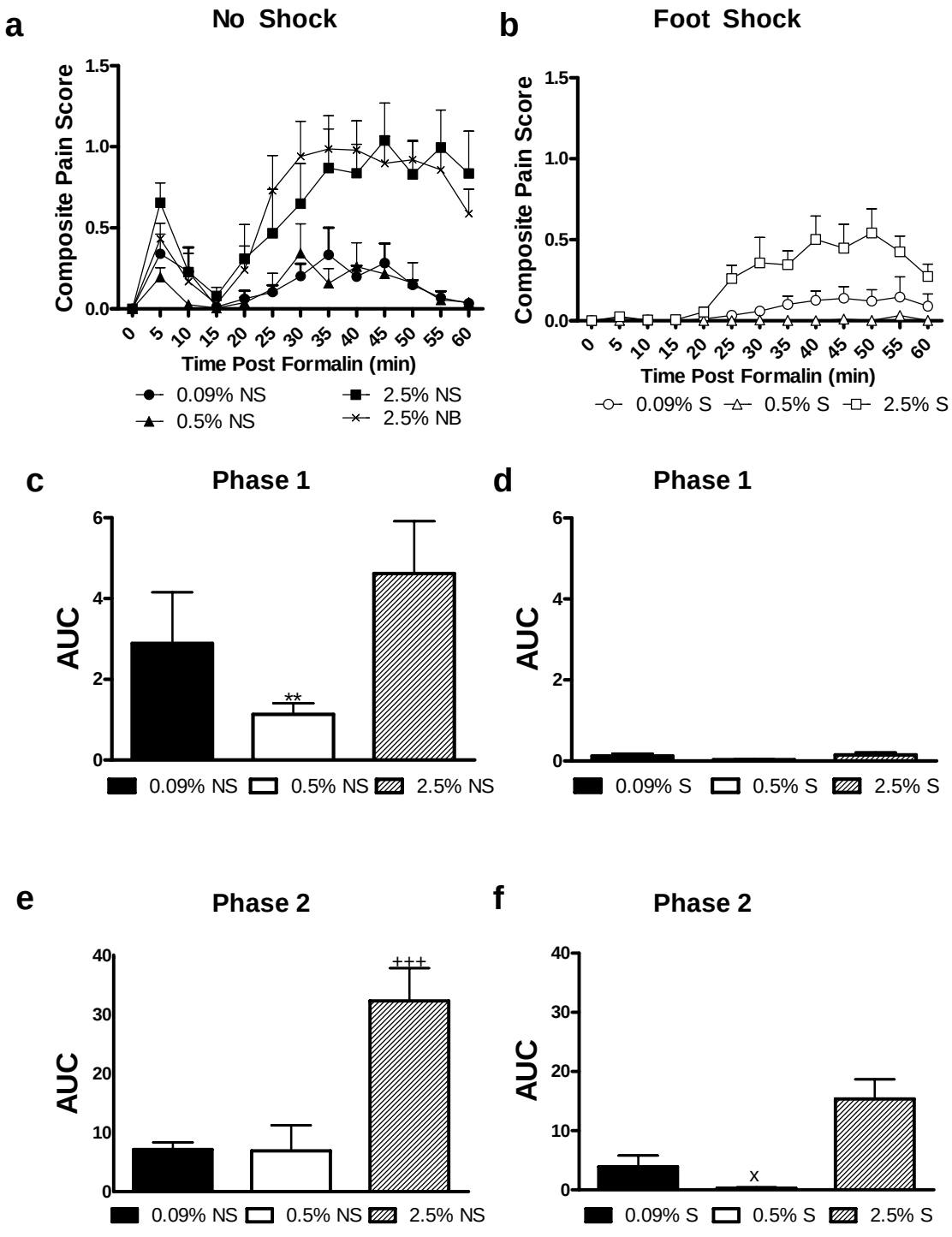
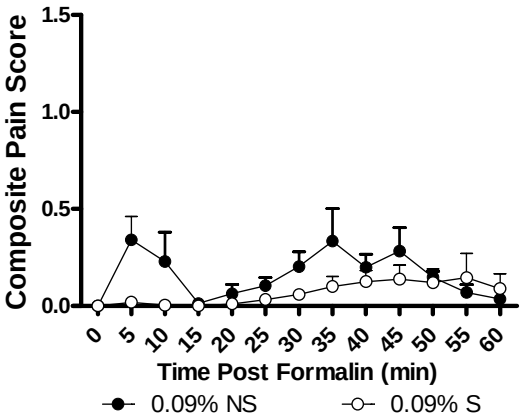

a
No Shock
Composite Pain Score
1.5
1.0
0.5
0.0
0 5 10 15 20 25 30 35 40 45 50 55 60
Time Post Formalin (min)
0.09% NS
0.5% NS
2.5% NS
2.5% NB
b
Foot Shock
Composite Pain Score
1.5
1.0
0.5
0.0
0 5 10 15 20 25 30 35 40 45 50 55 60
Time Post Formalin (min)
0.09% S
0.5% S
2.5% S
c
Phase 1
AUC
6
4
2
0
**
0.09% NS
0.5% NS
2.5% NS
d
Phase 1
AUC
6
4
2
0
0.09% S
0.5% S
2.5% S
e
Phase 2
AUC
40
30
20
10
0
+++
0.09% NS
0.5% NS
2.5% NS
f
Phase 2
AUC
40
30
20
10
0
x
0.09% S
0.5% S
2.5% S

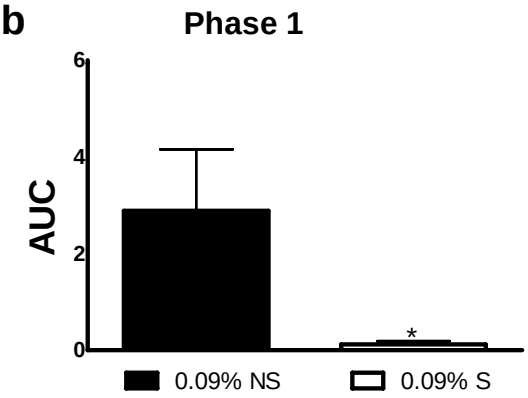
Figure 1. Exposure to foot shock stress reduces formalin-invoked pain behavior by producing SIA. Increasing the concentration of formalin increased pain behavior in rats subjected to non-shock (a) and foot shock (b) during both phases. NB curve matches NS curve. Early phase (c) and late phase (e) nociception in non-shocked rats was lower in rats receiving the two lower concentrations of formalin compared to 2.5% formalin. (d) Increasing the formalin concentration did not surmount the SIA-induced elimination of nociception during Phase 1. (f) 2.5% formalin was able to partially overcome SIA during the second phase. SIA (Stress-induced Analgesia), NS (No Shock), S (Shock), NB (No Shock Box Exposure).

Figure 2

a


Composite Pain Score
1.5
1.0
0.5
0.0
0
5
10
15
20
25
30
35
40
45
50
55
60
Time Post Formalin (min)
0.09% NS
0.09% S

b


Phase 1
AUC
6
4
2
0
*
0.09% NS
0.09% S

c

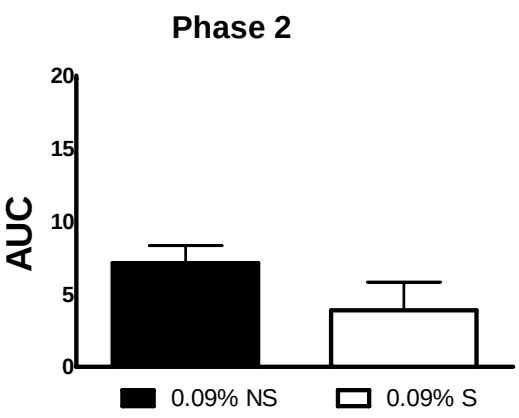
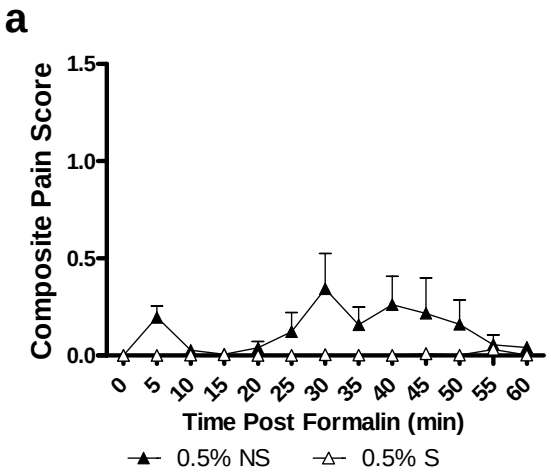

Phase 2
AUC
20
15
10
5
0
0.09% NS
0.09% S

Figure 2. SIA reduces pain behavior produced by 0.09% formalin. (b) Foot shock reduces nociception during Phase 1 (*$p < 0.05$). (c) Phase 2 pain behavior did not differ ($p = 0.144$) in S vs. NS rats. SIA (Stress-induced Analgesia), NS (No Shock), S (Shock).

Figure 3


a
Composite Pain Score
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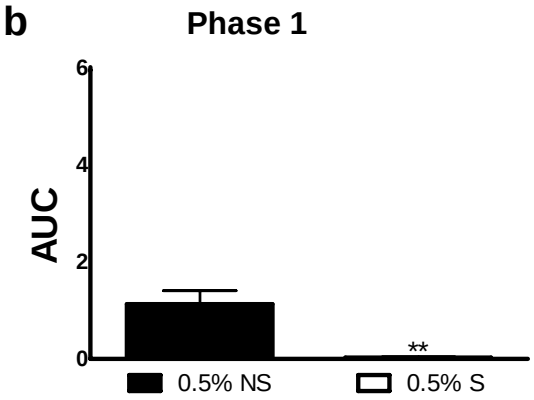
1.0
0.5
0.0
0 5 10 15 20 25 30 35 40 45 50 55 60
Time Post Formalin (min)
0.5% NS
0.5% S


b
Phase 1
AUC
6
4
2
0
**
0.5% NS
0.5% S

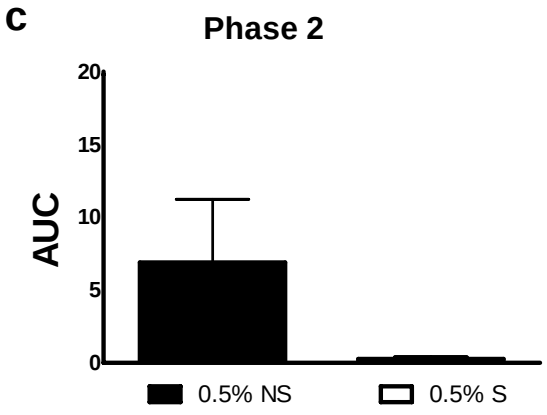
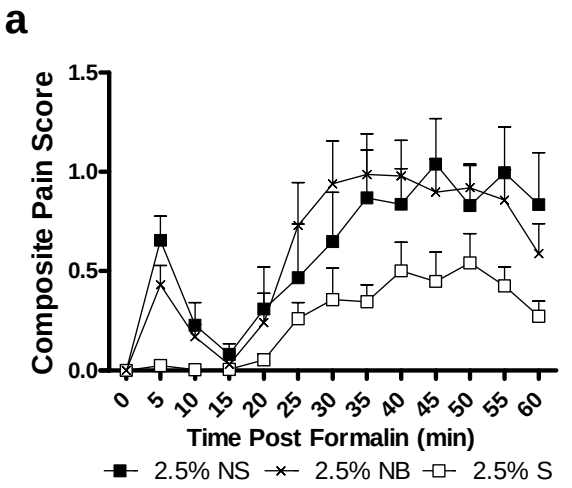
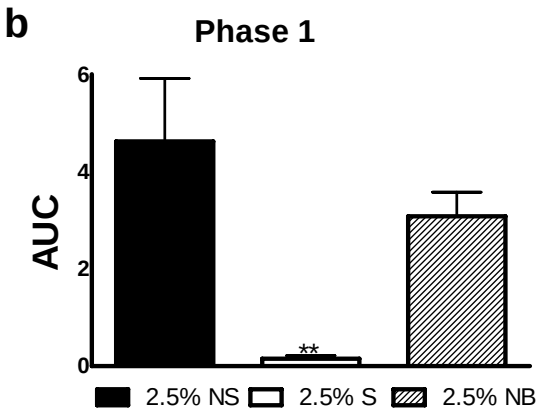

c
Phase 2
AUC
20
15
10
5
0
0.5% NS
0.5% S

Figure 3. SIA reduces pain behavior produced by 0.5% formalin. (b) Foot shock reduces nociception during Phase 1 (**$p < 0.01$). (c) Phase 2 pain behavior did not differ ($p = 0.0794$) in S vs. NS rats. SIA (Stress-induced Analgesia), NS (No Shock), S (Shock).

Figure 4


a
Composite Pain Score
1.5
1.0
0.5
0.0
0 5 10 15 20 25 30 35 40 45 50 55 60
Time Post Formalin (min)
2.5% NS
2.5% NB
2.5% S


b
Phase 1
AUC
6
4
2
0
**
2.5% NS
2.5% S
2.5% NB

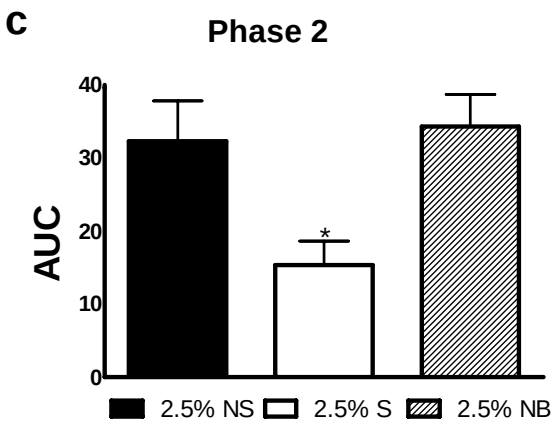
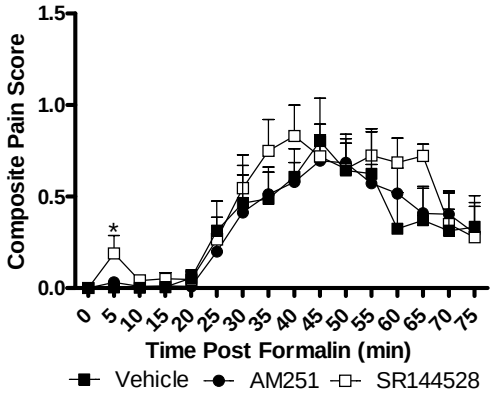

c
Phase 2
AUC
40
30
20
10
0
*
2.5% NS
2.5% S
2.5% NB

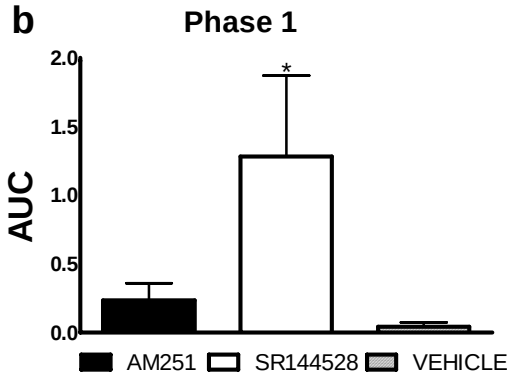
Figure 4. SIA reduces pain behavior produced by 2.5% formalin. (b) Foot shock reduces nociception during Phase 1 (**$p < 0.01$). (a)/(c) Phase 2 nociception was lower in S vs. NS rats (*$p < 0.05$). Controls not exposed to the footshock chamber (NB) did not differ from NS animals (P= 0.4765). SIA (Stress-induced Analgesia), NS (No Shock), NB (No Shock Box Exposure), S (Shock).

Figure 5

a


Composite Pain Score
1.5
1.0
0.5
0.0
*
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75
Time Post Formalin (min)
Vehicle
AM251
SR144528

b


Phase 1
AUC
2.0
1.5
1.0
0.5
0.0
*
AM251
SR144528
VEHICLE

c

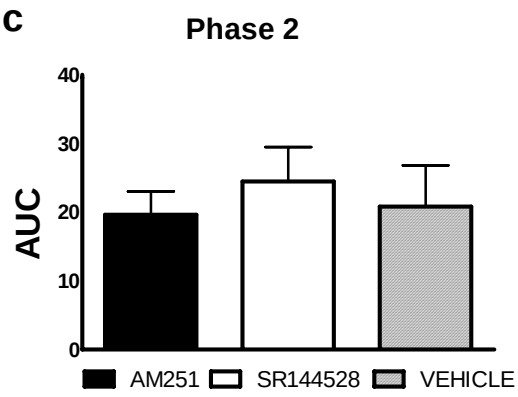
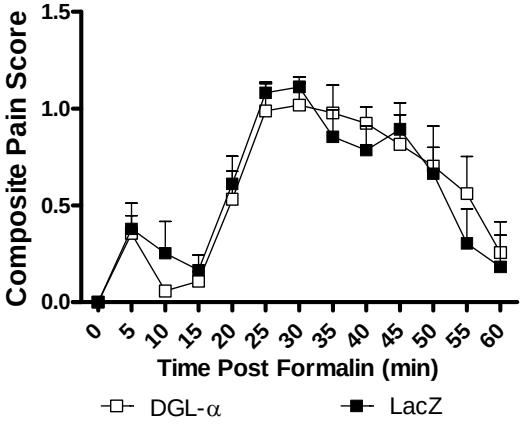

Phase 2
AUC
40
30
20
10
0
AM251
SR144528
VEHICLE

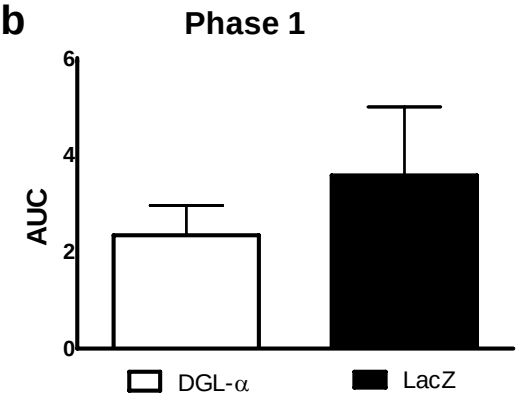
Figure 5. Effects of blockade of CB_1 (with AM251) and CB_2 (with SR144528) on SIA. (a) Local injection of the CB_2 antagonist SR144528, but not the CB_1 antagonist AM251, increased formalin-induced pain behavior relative to Vehicle. Drugs were administered to the paw 15 minutes prior to footshock and subsequent formalin injection (s.c.). (b) SR144528 increased pain behavior during Phase 1 ($*p < 0.05$) when compared to the vehicle, documenting a CB_2-mediated blockade of SIA. AM251 produced a trend ($p = 0.1053$) toward increased nociception when compared to the vehicle. (c) Second phase pain behavior did not differ between groups. SIA (Stress-induced Analgesia), s.c. (subcutaneous).

Figure 6

a


Composite Pain Score
1.5
1.0
0.5
0.0
0
5
10
15
20
25
30
35
40
45
50
55
60
Time Post Formalin (min)
DGL-α
LacZ

b


Phase 1
AUC
6
4
2
0
DGL-α
LacZ

c

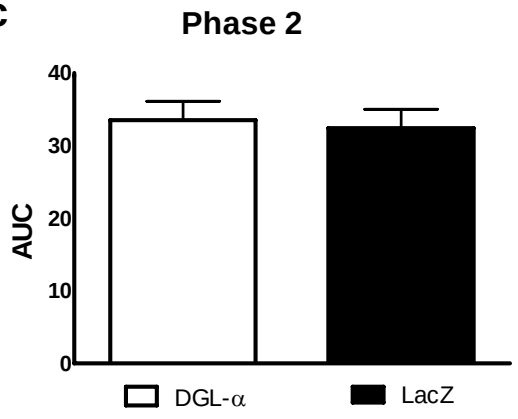

Phase 2
AUC
40
30
20
10
0
DGL-α
LacZ

Figure 6. Effects of a DGLα RNA-silencing viral vector. (a) Local injections of 2.5% formalin on NS animals yielded similar pain behavior in both groups ($p > 0.05$). (b)/(c) Pain behavior did not differ significantly during the first phase nor during the second phase. NS (No Shock).

Appendix

Fig. 1c

Table Analyzed	AUC 0-15			
One-way analysis of variance				
P value	0.0756			
P value summary	ns			
Are means signif. different? (P < 0.05)	No			
Number of groups	3			
F	3.170			
R square	0.3278			
Bartlett's test for equal variances				
Bartlett's statistic (corrected)	7.856			
P value	0.0197			
P value summary	*			
Do the variances differ signif. (P < 0.05)	Yes			
ANOVA Table	SS	df	MS	
Treatment (between columns)	33.28	2	16.64	
Residual (within columns)	68.25	13	5.250	
Total	101.5	15		
Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary
0.09% NS vs 0.5% NS	1.754	1.788	No	ns
0.09% NS vs 2.5% NS	-1.735	1.693	No	ns
0.5% NS vs 2.5% NS	-3.488	3.555	No	ns

Figure 1d

Table Analyzed	AUC 0-15			
One-way analysis of variance				
P value	0.2729			
P value summary	ns			
Are means signif. different? (P < 0.05)	No			
Number of groups	3			
F	1.410			
R square	0.1499			
Bartlett's test for equal variances				
Bartlett's statistic (corrected)	9.974			
P value	0.0068			
P value summary	**			
Do the variances differ signif. (P < 0.05)	Yes			

ANOVA Table	SS	df	MS	
Treatment (between columns)	0.04710	2	0.02355	
Residual (within columns)	0.2672	16	0.01670	
Total	0.3143	18		

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary
0.09% S vs 0.5% S	0.09202	1.810	No	ns
0.09% S vs 2.5% S	-0.02687	0.5285	No	ns
0.5% S vs 2.5% S	-0.1189	2.254	No	ns

Figure 1e

Table Analyzed	AUC 15-60
One-way analysis of variance	
P value	0.0012
P value summary	**
Are means signif. different? (P < 0.05)	Yes
Number of groups	3
F	11.81
R square	0.6449
Bartlett's test for equal variances	
Bartlett's statistic (corrected)	6.539
P value	0.0380
P value summary	*
Do the variances differ signif. (P < 0.05)	Yes

ANOVA Table	SS	df	MS
Treatment (between columns)	2196	2	1098
Residual (within columns)	1209	13	92.99
Total	3405	15	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary
0.09% NS vs 0.5% NS	0.2365	0.05728	No	ns
0.09% NS vs 2.5% NS	-25.14	5.830	Yes	**
0.5% NS vs 2.5% NS	-25.38	6.147	Yes	**

Fig 1f

Table Analyzed	AUC 15-60
One-way analysis of variance	
P value	0.0005
P value summary	***
Are means signif. different? (P < 0.05)	Yes
Number of groups	3
F	12.49
R square	0.6096
Bartlett's test for equal variances	
Bartlett's statistic (corrected)	22.71
P value	< 0.0001
P value summary	***
Do the variances differ signif. (P < 0.05)	Yes

ANOVA Table	SS	df	MS
Treatment (between columns)	750.4	2	375.2
Residual (within columns)	480.6	16	30.04
Total	1231	18	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary
0.09% S vs 0.5% S	3.610	1.674	No	ns
0.09% S vs 2.5% S	-11.47	5.319	Yes	**
0.5% S vs 2.5% S	-15.08	6.739	Yes	***

Fig. 2b

Table Analyzed	Copy of AUC 0-15
Column A	0.09% NS
vs	vs
Column B	0.09% S
Unpaired t test	
P value	0.0130
P value summary	*
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	One-tailed
t, df	t=2.611 df=10
How big is the difference?	
Mean ± SEM of column A	2.886 ± 1.274 N=5
Mean ± SEM of column B	0.1245 ± 0.06154 N=7
Difference between means	2.761 ± 1.057
95% confidence interval	0.4054 to 5.117
R square	0.4054
F test to compare variances	
F,DFn, Dfd	306.0, 4, 6
P value	< 0.0001
P value summary	***
Are variances significantly different?	Yes

Fig. 2c

Table Analyzed	Copy of AUC 15-60
Column A	0.09% NS
vs	vs
Column B	0.09% S
Unpaired t test	
P value	0.1140
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	One-tailed
t, df	t=1.284 df=10
How big is the difference?	
Mean ± SEM of column A	7.142 ± 1.204 N=5
Mean ± SEM of column B	3.889 ± 1.940 N=7
Difference between means	3.253 ± 2.533
95% confidence interval	-2.390 to 8.896
R square	0.1416
F test to compare variances	
F,DFn, Dfd	3.637, 6, 4
P value	0.2319
P value summary	ns
Are variances significantly different?	No

Fig. 3b

Table Analyzed	Copy of AUC 0-15
Column C	0.5% NS
vs	vs
Column D	0.5% S
Unpaired t test	
P value	0.0012
P value summary	**
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	One-tailed
t, df	t=4.012 df=10
How big is the difference?	
Mean ± SEM of column C	1.132 ± 0.2738 N=6
Mean ± SEM of column D	0.0325 ± 0.01217 N=6
Difference between means	1.099 ± 0.2741
95% confidence interval	0.4888 to 1.710
R square	0.6168
F test to compare variances	
F,DFn, Dfd	506.2, 5, 5
P value	< 0.0001
P value summary	***
Are variances significantly different?	Yes

Fig. 3c

Table Analyzed	Copy of AUC 15-60
Column C	0.5% NS
vs	vs
Column D	0.5% S
Unpaired t test	
P value	0.0794
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	One-tailed
t, df	t=1.523 df=10
How big is the difference?	
Mean ± SEM of column C	6.905 ± 4.349 N=6
Mean ± SEM of column D	0.2792 ± 0.1409 N=6
Difference between means	6.626 ± 4.351
95% confidence interval	-3.068 to 16.32
R square	0.1883
F test to compare variances	
F,DFn, Dfd	952.3, 5, 5
P value	< 0.0001
P value summary	***
Are variances significantly different?	Yes

Figure 4b

Table Analyzed	AUC 0-15			
One-way analysis of variance				
P value	0.0021			
P value summary	**			
Are means signif. different? (P < 0.05)	Yes			
Number of groups	3			
F	9.834			
R square	0.5842			
Bartlett's test for equal variances				
Bartlett's statistic (corrected)	21.89			
P value	< 0.0001			
P value summary	***			
Do the variances differ signif. (P < 0.05)	Yes			
ANOVA Table	SS	df	MS	
Treatment (between columns)	57.60	2	28.80	
Residual (within columns)	41.00	14	2.929	
Total	98.60	16		
Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary
2.5% NS vs 2.5% S	4.469	6.099	Yes	**
2.5% NS vs 2.5% NB	1.539	2.100	No	ns
2.5% S vs 2.5% NB	-2.930	4.194	Yes	*

Fig. 4c

Table Analyzed	AUC 15-60
One-way analysis of variance	
P value	0.0139
P value summary	*
Are means signif. different? (P < 0.05)	Yes
Number of groups	3
F	5.888
R square	0.4569
Bartlett's test for equal variances	
Bartlett's statistic (corrected)	0.7799
P value	0.6771
P value summary	ns
Do the variances differ signif. (P < 0.05)	No

ANOVA Table	SS	df	MS
Treatment (between columns)	1273	2	636.7
Residual (within columns)	1514	14	108.1
Total	2787	16	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary
2.5% NS vs 2.5% S	16.93	3.802	Yes	*
2.5% NS vs 2.5% NB	-2.024	0.4545	No	ns
2.5% S vs 2.5% NB	-18.95	4.464	Yes	*

Fig. 5.1b

Table Analyzed	AUC 0-15			
One-way analysis of variance				
P value	0.0681			
P value summary	ns			
Are means signif. different? (P < 0.05)	No			
Number of groups	3			
F	3.276			
R square	0.3188			
Bartlett's test for equal variances				
Bartlett's statistic (corrected)	23.71			
P value	< 0.0001			
P value summary	***			
Do the variances differ signif. (P < 0.05)	Yes			

ANOVA Table	SS	df	MS	
Treatment (between columns)	5.109	2	2.555	
Residual (within columns)	10.92	14	0.7799	
Total	16.03	16		

Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary
VEHICLE vs AM251	-0.1912	0.3576	No	ns
VEHICLE vs SR144528	-1.240	2.319	No	ns

Fig 5.2b

Table Analyzed	Copy of AUC 0-15
Column I	SR144528
vs	vs
Column J	VEHICLE
Unpaired t test	
P value	0.0448
P value summary	*
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	One-tailed
t, df	t=1.901 df=9
How big is the difference?	
Mean ± SEM of column I	1.283 ± 0.5895 N=6
Mean ± SEM of column J	0.04267 ± 0.03090 N=5
Difference between means	1.240 ± 0.6523
95% confidence interval	-0.2352 to 2.716
R square	0.2866
F test to compare variances	
F,DFn, Dfd	436.7, 5, 4
P value	< 0.0001
P value summary	***
Are variances significantly different?	Yes

Fig 5.3b

Table Analyzed	Copy of AUC 0-15
Column H	AM251
vs	vs
Column I	SR144528
Unpaired t test	
P value	0.0562
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	One-tailed
t, df	t=1.740 df=10
How big is the difference?	
Mean ± SEM of column H	0.2339 ± 0.1258 N=6
Mean ± SEM of column I	1.283 ± 0.5895 N=6
Difference between means	-1.049 ± 0.6027
95% confidence interval	-2.392 to 0.2939
R square	0.2325
F test to compare variances	
F,DFn, Dfd	21.95, 5, 5
P value	0.0041
P value summary	**
Are variances significantly different?	Yes

Fig. 5c

Table Analyzed	AUC 15-60			
One-way analysis of variance				
P value	0.7515			
P value summary	ns			
Are means signif. different? (P < 0.05)	No			
Number of groups	3			
F	0.2916			
R square	0.03999			
Bartlett's test for equal variances				
Bartlett's statistic (corrected)	1.008			
P value	0.6041			
P value summary	ns			
Do the variances differ signif. (P < 0.05)	No			

ANOVA Table	SS	df	MS	
Treatment (between columns)	76.15	2	38.08	
Residual (within columns)	1828	14	130.6	
Total	1904	16		

Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary
VEHICLE vs AM251	1.164	0.1682	No	ns
VEHICLE vs SR144528	-3.685	0.5326	No	ns

Fig. 6b

Table Analyzed	Copy of AUC 0-15
Column K	DGL-α
vs	vs
Column L	LacZ
Unpaired t test	
P value	0.2199
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	One-tailed
t, df	t=0.8044 df=10
How big is the difference?	
Mean ± SEM of column K	2.339 ± 0.6114 N=6
Mean ± SEM of column L	3.578 ± 1.415 N=6
Difference between means	-1.240 ± 1.541
95% confidence interval	-4.674 to 2.194
R square	0.06077
F test to compare variances	
F,DFn, Dfd	5.355, 5, 5
P value	0.0893
P value summary	ns
Are variances significantly different?	No

Fig. 6c

Table Analyzed	Copy of AUC 15-60
Column K	DGL-α
vs	vs
Column L	LacZ
Unpaired t test	
P value	0.3854
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	One-tailed
t, df	t=0.2994 df=10
How big is the difference?	
Mean ± SEM of column K	33.51 ± 2.616 N=6
Mean ± SEM of column L	32.40 ± 2.621 N=6
Difference between means	1.108 ± 3.703
95% confidence interval	-7.141 to 9.358
R square	0.008882
F test to compare variances	
F,DFn, Dfd	1.004, 5, 5
P value	0.9970
P value summary	ns
Are variances significantly different?	No

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