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Fear Conditioning and Extinction: Examining the Role of GSK3 β ser 389

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**Fear Conditioning and Extinction:
Examining the Role of GSK3 β ser 389**

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Abstract

GSK3 β is a serine threonine kinase that has been shown to influence numerous biological and psychological interactions, including the regulation of cell survival and cell death, as well as influencing mood disorders such as major depressive disorder and bipolar disorder. Preliminary data from our lab showed an exaggerated conditioned fear response in homozygous GSK3 β knock-in (GSK3 β KI) mice, which lacked the ability to phosphorylate GSK3 β at the ser 389 site due to a serine to alanine substitution. Based on heightened fear responses previously observed in our lab, we predicted that increased expression GSK3 β would result in a prolonged and heightened fear response, as GSK3 β expression would interfere with the ability to turn off fear of a conditioned stimulus. These mutants were given five tone plus shock fear conditioning trials, followed by six days of tone alone fear extinction training. In contrast to our preliminary data, GSK3 β KI mice did not show exaggerated conditioned fear, and showed no significant differences to wild type mice in fear extinction. To examine if these results were influenced by the age of the mice, a second study was conducted using two different age subsets of GSK3 β KI mice. The results demonstrated that there were no significant differences in fear acquisition or extinction based on age.

Introduction

Traumatic experiences can have devastating effects long after the initial trauma itself. Traumatic fear conditioning can take place as a result of natural disasters, serious accidents, physical and psychological abuse, and wartime experiences. Over time, the concept of posttraumatic stress disorder (PTSD) was introduced, which began to explore how fear can last for years after even a single traumatic event. Psychologically, it is understood that the fear experienced during trauma can become associated with a specific stimulus, such as a sound or an image. Learning to recognize and avoid these triggers can help to reduce the impact of PTSD. However, there are also physiological responses that modulate PTSD, and by better understanding the neurobiological mechanisms in place, we can develop better treatments to negate these harmful effects. Recent research in our lab suggests that glycogen synthase kinase 3 β (GSK3 β) may influence these neurobiological mechanisms to cause exaggerated fear in mice.

Functions of GSK3 β

GSK3 β is a serine threonine kinase that has been implicated in having an active role in numerous and varied biological and psychological processes. A relatively rare feature found in GSK3 β , (when compared to other kinases), is that this specific kinase is constitutively active. It is common for kinases to be inactive until being activated by phosphorylation, but in GSK3 β the reverse is true: the phosphorylation of GSK3 β at specific binding sites will inactivate this kinase. Prior research on this kinase had focused primarily on the inactivation of GSK3 β

at the ser 9-position (Cross et al., 1995). This inactivation was accomplished by the phosphorylation of ser 9 by protein kinase B and occurs largely in the cytoplasm of neurons.

One previously studied interaction is the role of GSK3 β in the cell death/cell survival signaling pathway. GSK3 β was found to often induce apoptosis in target cells, while other times promoting cell survival (Maurer et al., 2014). These researchers noted that PI3K, which represses cell death, also inhibits GSK3 activity, suggesting a possibly connection between GSK3 and cell death regulation. Indeed, this study suggested that GSK3 is either directly or indirectly a regulator of the Bcl-2 family proteins, which serve as anti-apoptotic factors, though its exact mechanism for this is currently unknown. Additionally, it was demonstrated that the lack of active GSK3 β in heterozygous GSK3 β knock-out mice (GSK $^{+/-}$) was associated with impaired memory reconsolidation in contrast to wild-type mice (Kimura et al., 2008). Since the presence of GSK3 β appears to be required for memory, this could suggest that overexpression of GSK3 β would make it difficult to forget or extinguish memories.

Behaviorally, some studies have also suggested that GSK3 β may play a central role in mood disorders including major depressive disorder and bipolar disorder. One such study demonstrated that environmental and genetic factors can result in high levels of GSK3, leading to mood destabilization from decreased axonal growth and neurotransmitter release (Beurel et al., 2012). Additionally, lithium, (which is commonly used as a treatment for bipolar disorder), directly inhibits GSK3, suggesting the possibility that this inhibition may be the

mechanism for lithium's effectiveness (O'Brien and Klein, 2009). As a result, there is interest in examining whether GSK3 β inactivation may be effective as a therapy for varying mood disorders (Aricioglu and Gumru, 2013).

Though there is a growing understanding of how GSK3 β impacts apoptosis, memory, and mood disorders (among other functions), it is not known how (if at all) these functions are related to each other. One study on post-traumatic stress disorder (PTSD) began to suggest a link between GSK3 β expression and fear. In this study, PTSD-susceptible mice and PTSD-resilient mice were subjected to a footshock and were tested 42 days later for changes in brain structure (Dahlhoff, et al., 2010). In comparison to the resilient mice, the PTSD-susceptible mice were shown to have increased levels of phosphorylated GSK3 β in the basolateral amygdala, a brain region well documented for its involvement in conditioned fear. Interestingly, these results suggest that the inactivation of GSK3 β , not its activation, may result in prolonged and/or exaggerated fear.

While the previous studies focused on the phosphorylation of GSK3 β at the serine-9 position, a novel regulatory site of GSK3 β was more recently discovered at the serine-389 position. The phosphorylation of this site by p38 mitogen-activated protein kinase (p38 MAPK) and occurs largely in the nucleus of the neuron (Thornton et al., 2008). This finding is significant, as previous methods for manipulating GSK affected either the entire protein or the cytosolic protein, both of which are expressed throughout the body. By identifying a

phosphorylation site mainly localized to the neuron, we can better examine how nuclear GSK expression affects neuronal cell death and behavioral phenotypes.

GSK3 β and fear

Though p38 MAPK has the ability to inhibit GSK3 β , there is another method of releasing the inhibition on GSK3 β . By swapping an alanine for the serine at the 389 position of GSK3 β , p38 is prevented from phosphorylating the site, leaving it GSK3 β constitutively active (Thornton et al., 2008). This alteration produced important psychological distinctions between the GSK3 β KI mice and the wild-type mice. While visually appearing identical to normal mice, the GSK3 β KI mice were more prone to flee from nearby motion, as well as being more likely to bite and/or rattle their tails during attempts to pick them up. Some mice also were documented biting at the tails of siblings in their cages.

Preliminary data from our lab noted that the GSK3 β KI mice appeared to exhibit an overgeneralized fear response to fear conditioning, while displaying normal anxiety and normal maze learning. After being conditioned to fear a certain context, both GSK3 β KI and wild-type mice exhibited a significant fear response (measured by the amount of time frozen in place) when placed back in the training context. However, GSK3 β KI mice were shown to have a greater degree of freezing behavior than the wild-type mice. Perhaps even more significantly, the GSK3 β KI mice also exhibited a high degree of freezing after fear conditioning when placed in a novel context, while wild-type mice had a

minimal response to a different context. This suggests that GSK3 β KI mice experienced an overgeneralization of fear across contexts (Hare, 2015).

Similar experiments focused on the fear exhibited to a specific tone instead of to a specific context. In these experiments, a significant difference between the GSK3 β KI mice and the wild type mice was recorded. First, GSK3 β KI and wild-type mice were conditioned to fear a specific tone. When this tone was played later in a new context, both GSK3 β KI and wild-type mice initially froze to that tone. However, the wild-type mice resumed movement after a few minutes, while the GSK3 β KI continued to freeze (Hare, 2015). Taken together, these results from our lab suggest that the overexpression of GSK3 β may result in both increased acquisition and sustained duration of a conditioned fear response.

One other intriguing finding from our recent work is the effect GSK3 β may have on neuronal cells. While researching the behavioral effects of GSK3 β , neuronal degradation was found in the hippocampal and cortical regions of the brains of GSK3 β knock-in mice (Hare, 2015). This may help to explain why a heightened fear phenotype may be present. The hippocampus has a prominent role in emotion and memory, while the cortex (particularly the prefrontal cortex) can help to regulate brain regions such as the amygdala. With neuronal degradation decreasing the function of these regions, important regulation of the fear and memory centers may be lost.

The purpose of this study is to better understand the contribution of glycogen synthase kinase 3 β (GSK3 β) in conditioned fear, a procedure that models aspects of posttraumatic stress disorder. Previous research has suggested

that the inability to inhibit this kinase may be associated with an enhanced conditioned fear response in mice. This study was designed to examine two hypothesized reasons to explain the previously observed increases in fear responses: 1) active GSK3 β may enhance the acquisition of a fear response, producing higher initial levels of fear in comparison to wild-type mice, and/or 2) active GSK3 β may interfere with the process of fear extinction, prolonging an acquired fear response for an extended duration.

Methods (Experiment 1)

Subjects

Homozygous GSK KI mice were bred in house from breeding stock obtained from Mercedes Rincon, Department of Immunology University of Vermont. GSK KI mice were on a C57BL6/J background. Age-matched wild type mice were purchased from Jackson Laboratory (Bar Harbor Main). A total of 18 GSK KI and 20 wild type mice were used in the study. Mice were male and 8 weeks of age at the start of the study. Mice were housed in groups of 4 to 5 in standard acrylic cages in a conventional animal facility. Food and water were available ad lib and the mice were on a 12-hour light dark cycle with lights on at 7 am.

Apparatus

All training and testing was performed in commercially available fear conditioning boxes (Med Associates, St. Albans Vermont), though each box was modified into two distinct contexts: Context A for tone-shock pairing, and context B for fear extinction trials. The chambers in both contexts were inside sound attenuating cubicles. Context A was a darkened box with metallic walls, grid floor, and a nearby paper towel coated in Vicks VapoRub. For context B, an insert was placed in the box such that the floor was white and smooth and the walls made of cardboard. The smell of Vicks VapoRub from Context A was replaced by placing a paper towel coated in 2% anise extract solution next to the box. The lights in Context B were also turned on. For both contexts, an infrared camera was placed facing the box with the mouse inside, which was set up to record changes

in movement once testing began by determining the number of pixel changes in the image.

Procedure

Tone and shock fear conditioning was carried out on day 1. Mice were placed individually into Context A and after a two-minute acclimation period, presented with the first of 5 tone and shock trials at a 30 sec inter trial interval. Each trial consisted of a 75 dB, 4500 Hz tone for 30 seconds, ending with a 1 second 0.3 mA footshock. At the end of conditioning, mice were returned to the colony room.

Extinction training was carried out on each of the next six days. For each extinction training day, mice were placed in context B. After a two-minute acclimation period, the mice were given a continuous three-minute tone. This procedure was repeated daily for a total of six days.

Freezing was measured with Video Freeze Software (Med Associates, St. Albans, Vermont) using procedures published by Anagnostaras et al., 2010.

Data Reduction and Analysis

The total percentage of time spent freezing to the tone over the 3-minute extinction trial was recorded for each day of extinction training. These data were subject to ANOVA, with genotype as a between subjects factor and extinction as a within subjects factor. For experiment one, a total of 18 GSK3 β KI and 20 wild type mice were used.

Results (Experiment 1)

Figure 1 shows the results of the six days of fear extinction training.

Overall, WT and KI both showed extinction over the six sessions. However, the differences in freezing behavior between the two groups were not enough to attain significance ($F(5,165)=1.156, p=.333$).

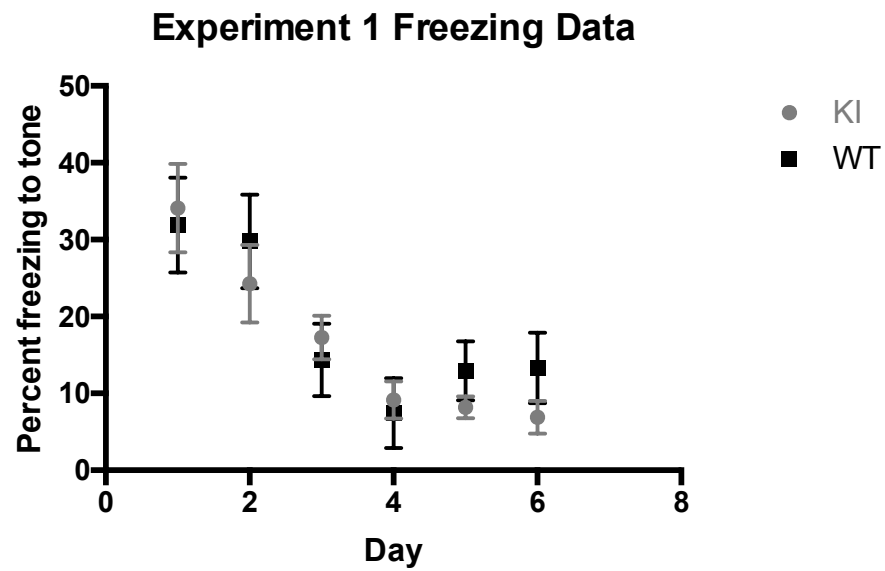


Figure 1: Experiment 1 freezing trends

Experiment 2

The results of the first experiment failed to replicate the exaggerated fear conditioning that had previously been observed in our lab. However, previous work in our lab had used relatively older mice in comparison to the 8-10 week old mice used in Experiment 1. We hypothesized that one possible explanation for the behavioral differences from past experiments might be the age of the animals. In particular, we believed that the neuronal degradation that was found in the hippocampal and cortical regions of GSK3 β KI mice (Hare, 2015), may be related to accumulated stress, thus eliciting a stronger response over time.

Methods (Experiment 2)

Subjects

Overall, twenty-one GSK3 β KI and twenty wild-type mice were tested. Of the GSK3 β KI mice, ten were 17-20 weeks old, and eleven were 10-12 weeks old. For the wild types, eight were 19 weeks old and twelve were 8-11 weeks old. Housing and all other conditions were the same as Experiment 1.

Testing Apparatus

Same as Experiment 1

Procedure

Same as Experiment 1

Data Reduction and Analysis

Same as Experiment 1

Results (Experiment 2)

Figure 2 shows the results of the six sessions of extinction. Overall WT mice showed greater extinction to the tone than KI mice ($F(5,185)=3.542$, $p=.004$). However, this was most likely the result of WT mice showing greater freezing to the tone on extinction day 1 ($t(39)=2.151$, $p=.038$). Both WT and KI mice showed extinction over the six sessions ($F(5, 100)=9.506$, $p=.000$; $F(5,95)=14.042$, $p=.000$, KI and WT respectively).

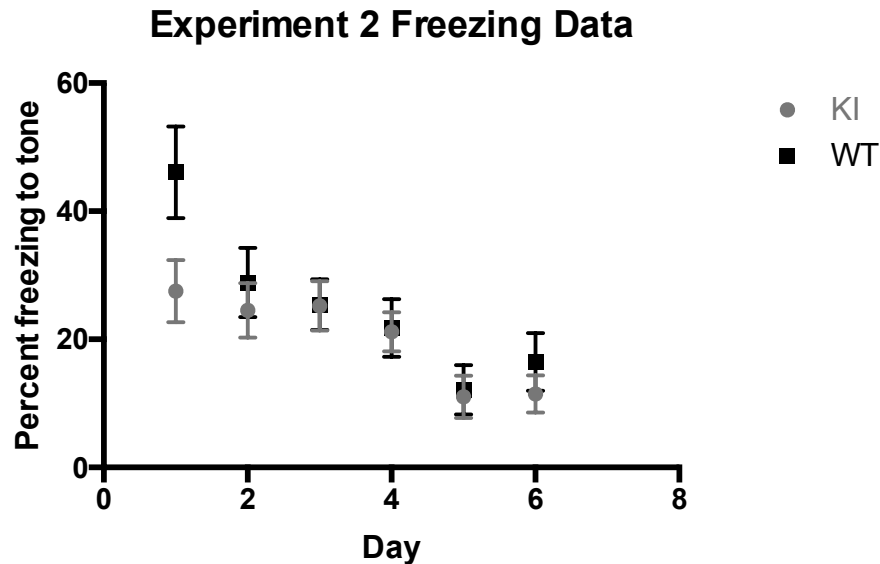


Figure 2: Experiment 2 freezing trends

Figures 3 and 4 demonstrate that although a moderate increase in fear was found in older mice in comparison to younger, age was not found to be a significant factor between the KI and WT mice ($F(5, 185)=.929$, $p=.464$).

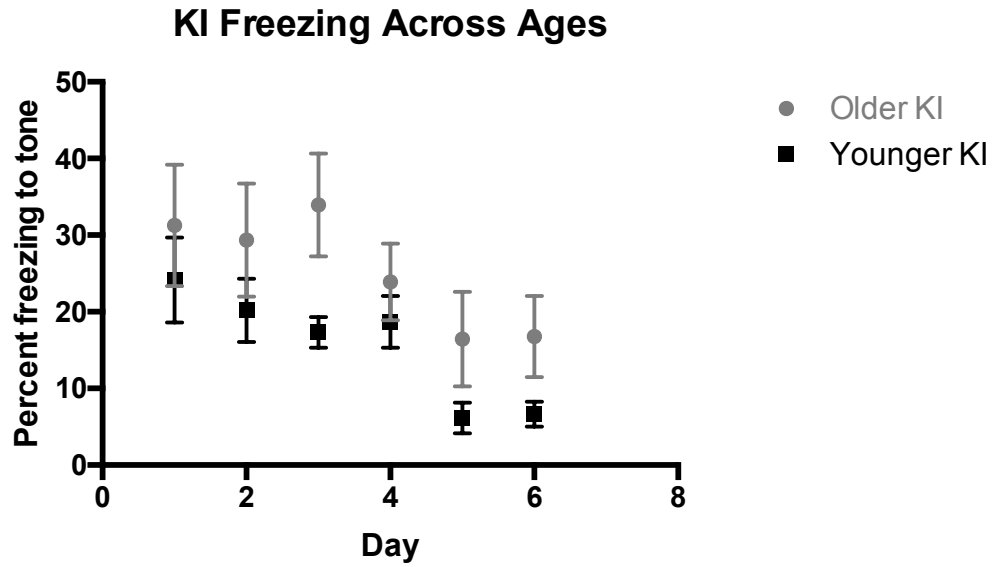


Figure 3: KI freezing differences based on age

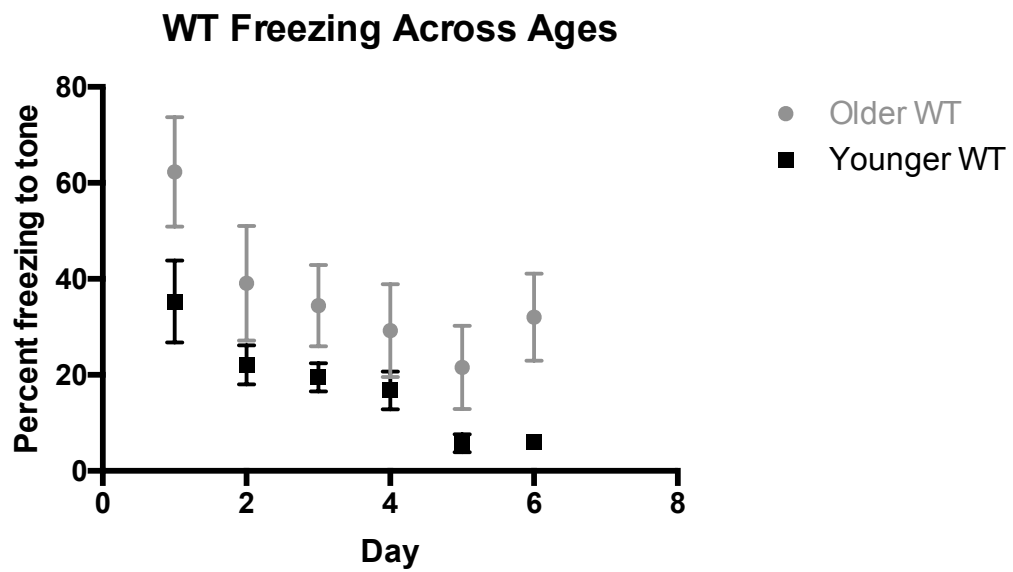


Figure 4: WT freezing differences based on age

Discussion

Overall, no significant differences were found between the GSK3 β knock-ins and the wild type mice. Though consistent fear responses were elicited to the tone stimulus, the initial amount of freezing, while varying greatly among individual mice, was demonstrated on average to be similar between GSK3 β KI and WT mice. Additionally, the rate of fear extinction over the six-day extinction training period was also similar between both cohorts of mice.

During the second experiment, two age groups were run to see if GSK3 β KI mice exhibited any differences in response based on age. While age was considered as a possible factor in the ability to extinguish fear, there had been no evidence to date linking the two. While the results from this replication did indicate a moderate increase in the initial level of fear for older KI mice, the older wild-types run as a control similarly exhibited an increased initial level of fear. Within each group, the rate of fear extinction was nearly identical between older and younger mice. Thus, the GSK3 β knock-ins did not display any significant differences in either acquisition of fear or extinction of fear based on age.

In Experiment 2, the only significant result was found to be between the average freezing between KI and WT mice, regardless of age. This is in contrast to the results of Experiment 1, where no significance was achieved. However, it is most likely that the significance from Experiment 2 is a result of the heightened fear response in the wild type mice on day 1 of extinction training. While it is theoretically possible that the trends observed could indicate that the KI mice are demonstrating a decreased rate of fear extinction in comparison to the wild types,

this result more likely demonstrates an increased fear acquisition in the wild types.

As often happens when conducting scientific experiments, the results from this study have generated more questions than from the beginning. Chief among those questions is why there is a discrepancy between these results (in not finding a conclusive link between GSK3 β and either fear acquisition or extinction) and from previous work in the Falls lab, which had found what appeared to be a connection between GSK3 β and fear.

One possible explanation is that the premise of this experiment is based off of a type I error. As stated previously, many individual mice (in both the GSK3 β KI and wild-type groups) displayed great variability in both their acquisition of the fear response as well as their relative rate of fear extinction. Indeed, early in the process of conducting Experiment 1, (albeit with a small number of mice run at this point), there appeared to be a clear difference between the GSK3 β knock-ins and the wild types. It is plausible that the previous work in this lab noticed a fear response that is nothing more than statistical variability in this behavioral response, and that on average there is no significant difference between GSK3 β knock-ins and wild types where fear is concerned.

There is also a confounding factor that we later noted that may have influenced the results. Though care was taken to ensure the conditions of the experiment itself were consistent for all mice tested, the same cannot be said for the upbringing of said mice. In years past, both the GSK3 β KI mice and the wild-type mice had been sourced from another lab. At the beginning of this

experiment, multiple GSK3 β breeding pairs were created meaning that all GSK3 β mice used in this experiment were born and raised in the same colony room.

Unfortunately, the wild types used in this experiment were purchased and not bred in house. The process of shipping the mice across multiple states, followed by the potential stressors of different types of bedding, different cages, and different room environment (among others) may have introduced a confounding variable. Tuli et al. (1995) found that normal mouse behaviors such as climbing, grooming, feeding, and sexual activity all underwent significant changes after transportation, with acclimatization often lasting more than four days. It is therefore possible that the transportation of these mice activated a long-term stress pathway that caused a heightened fear response during testing. Future studies in this area should ensure that the upbringing conditions be similar for all groups of mice involved in the testing.

While not explaining the discrepancy between previous and current data regarding GSK3 β KI extinction trends, it may also be beneficial in future studies to find a way to selectively activate GSK3 β in specific areas of the brain. Both of the methods used in the Falls lab (either knocking in the GSK3 β gene or knocking out its inhibitor, p38) result in GSK3 β being constitutively active throughout the entire brain and body. Thus, any effects (or lack thereof) that we observe may be influenced by how GSK3 β interacts with multiple body systems. If methods were developed to selectively activate GSK3 β in, say, the amygdala or the prefrontal cortex, we may be able to learn more about how GSK3 β might play a role in the learning or extinguishing of fear. On a related track, it may also prove useful to

stain brain sections to determine where GSK3 β is active in normal mice, and attempt to determine what conditions would cause the gene to be more or less active than usual.

Acknowledgements

I would like to thank my research advisor, Bill Falls, for his counsel and support. It was Bill who secured my interest in biopsychology and welcomed me with open arms when I expressed interest in joining his lab. Without Bill, I would not have had this amazing research experience, let alone my psychology minor itself. His dedication to all of the students in the lab continues to amaze me despite his busy schedule, and it will always be remembered with gratitude.

I would also like to thank both graduate students who I have had the pleasure to work with, Brendan Hare and Stephanie Menotti. Under their guidance, I was able to better understand the conceptual nature of the work being done, as well as honing my practical skills in facilitating the research. I would like to extend my gratitude to all of the other undergraduate students in the Falls lab who have come together and assisted me throughout the entirety of this project.

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