# Transcriptional regulation of the serotonin 1A receptor by lithium:
## roles of glycogen synthase kinase 3β and Deaf-1

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# Abstract

Serotonin 1A receptors ($5HT_{1A}$) located on serotonin neurons are the brakes on the serotonin system. Upregulated expression of these inhibitory autoreceptors has been implicated in resistance to antidepressant treatment, as well as being associated with depression and suicide in its own right. Because $5HT_{1A}$ heteroreceptors are, conversely, important in antidepressant response, proteins that are able to differentially regulate pre- and post-synaptic $5HT_{1A}$ are attractive antidepressant drug targets. The transcription factor deformed epidermal autoregulatory factor 1 (Deaf-1) is one such protein: acting as a presynaptic repressor and postsynaptic enhancer of $5HT_{1A}$ transcription. Because Deaf-1 has been shown to interact with glycogen synthase kinase 3β (GSK3β) — a constitutively active protein kinase that is inhibited by the mood stabilizer lithium at therapeutic concentrations — in vitro, we investigated whether these two proteins might interact to regulate the transcription of $5HT_{1A}$. In HEK293 cells containing a $5HT_{1A}$ promoter luciferase reporter construct, overexpression of Deaf-1 reduced $5HT_{1A}$ promoter activity by ~45%. Both lithium and the selective GSK3 inhibitor CHIR-99021 concentration-dependently attenuated this repression compared to matched controls. The results of a GSK3β reporter construct showed that this effect was indeed associated with inhibition of GSK3β for both drugs. Overall, these results support the hypothesis that GSK3β regulates the activity of Deaf-1 and, by proxy, $5HT_{1A}$ transcription.

# Introduction

## A. Depression

Major depressive disorder (depression) is a debilitating psychiatric disorder with one-year and lifetime prevalences of ~7% and 16.2%, respectively, in the United States[1,2]. Depression is chiefly characterized by low mood and loss of the ability to find pleasure in life (anhedonia), but other possible symptoms such as impaired concentration and fatigue contribute towards the burden presented by this disorder. In fact, this condition is expected to become the leading contributor to the global burden of disease by the year 2030, according to the World Health Organization[3].

## B. Serotonin and the treatment of mood disorders

All known antidepressants include strengthening of monoaminergic tone in the forebrain as a core pharmacological feature, and this is believed to underlie their therapeutic effects[4*]. The very first generation of antidepressants, the monoamine oxidase inhibitors, did this by inhibiting the oxidative degradation of dopamine, norepinephrine, and serotonin. The tricyclic and tetracyclic antidepressants that followed potently inhibited the reuptake of norepinephrine and serotonin, causing these monoamines to remain in the synapse for a longer period, and consequently increasing their effects.

Over time both basic research and clinical strategies have increasingly emphasized the role of serotonin in depression. This is partly because of the abundance of evidence connecting abnormalities in the serotonin system to depression in humans[7–14] and because of the overall

[*] Even the NMDA antagonist ketamine – an unusual fast-acting antidepressant[5] – has recently been shown to acutely increase the firing rate and/or population activity of monoaminergic neurons *in vivo*[6].

success of selective serotonin reuptake inhibitors (SSRIs) in the treatment of depression[15,16]. However, the majority of patients are obliged to trial multiple modern antidepressants before experiencing relief of their depressive symptoms[17]. When the 2-6 week latency before any benefit is usually seen with reuptake inhibitors is considered, it becomes clear that the need for improved and especially faster-acting antidepressant drugs is substantial.

## C. Antidepressant latency and $5HT_{1A}$

Pre-synaptic $5HT_{1A}$ receptors are the brakes on the serotonin system. Elevated expression of this population of $5HT_{1A}$ is associated with increased risk to attempt suicide[13,14†], and has even been proposed as a potential biomarker of depression in its own right[12]. Somatodendritic $5HT_{1A}$ autoreceptors located on the serotonin neurons of the dorsal raphe nucleus – from which the serotonergic projections throughout the forebrain originate – serve to detect local serotonin release and respond by downregulating serotonin synthesis and depressing neuronal firing rate[18]. SSRIs indiscriminately potentiate serotonin signaling in the forebrain and the dorsal raphe; an acute dose of SSRI therefore leads to immediate homeostatic adaptation of serotonergic tone mediated by these $5HT_{1A}$ autoreceptors[19]. The result is that until presynaptic $5HT_{1A}$ become desensitized by chronic treatment, SSRIs are not able to increase serotonin signaling. The time taken for this desensitization to occur is believed to account for the latency period during which these drugs do not produce a therapeutic effect.

† Within the subgroup of patients who attempted suicide, higher levels of $5HT_{1A}$ autoreceptors have been further correlated with increased lethality.

Because post-synaptic $5HT_{1A}$ receptors are, conversely, implicated in *response* rather than resistance to antidepressant treatment[18‡], simply administering an SSRI along with a $5HT_{1A}$ antagonist is not a viable strategy to treat depression. However, multi-modal antidepressants that incorporate a measure of $5HT_{1A}$ agonism as well as serotonin reuptake inhibition into their pharmacodynamics have recently been developed with some success. Theoretically, these drugs (vilazodone and vortioxetine) hasten the desensitization of pre-synaptic $5HT_{1A}$ without excessively blocking the post-synaptic receptors, leading to a somewhat faster onset of action[19,20].

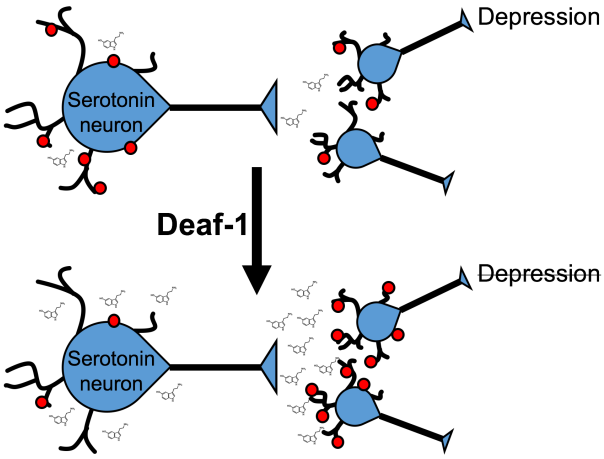


**Figure 1. Deaf-1 conceptualized as an endogenous antidepressant.** The transcription factor Deaf-1 pre-synaptically represses and post-synaptically enhances expression of $5HT_{1A}$ (red circles). This leads to increased serotonin synthesis and firing rate of serotonin neurons, as well as increasing the post-synaptic response to this monoamine. Strengthened serotonergic tone modulates the activity of circuits involved in regulation of affect, improving symptoms of depression.

## D. Re-balancing the serotonin system

A drug that somehow selectively blocks the action of pre-synaptic $5HT_{1A}$ would upregulate the activity of the serotonin system as a whole (via disinhibition), and do so without the need for the protracted desensitization process associated with conventional antidepressants[18,20]. The viability of such a strategy is supported by analogy with mirtazapine[21]. Mirtazapine is a

‡ It is important to note that whereas pre-synaptic $5HT_{1A}$ become desensitized by chronic treatment with SSRIs, post-synaptic $5HT_{1A}$ appear to be more resistant. Consequently, the ratio of pre- to post- synaptic $5HT_{1A}$ decreases over the first few weeks of antidepressant treatment.

somewhat unique antidepressant in that it has no appreciable affinity for any reuptake transporter, but is instead believed to act via blockade of inhibitory $\alpha_2$-adrenoreceptors. $\alpha_2$ are expressed as inhibitory autoreceptors on the norephinephrine neurons of the locus coeruleus (responsible for the forebrain projections of the noradrenergic system), and as inhibitory heteroreceptors on the serotonin neurons of the dorsal raphe. Mirtazapine is therefore thought to rapidly increase the release of both noradrenaline and serotonin, and is believed to have a faster onset of action than do SSRIs[§].

Because it is not possible to develop a selective ligand for pre-synaptic $5HT_{1A}$, proteins that are able to differentially regulate pre- and post-synaptic $5HT_{1A}$ are attractive drug targets. The transcription factor deformed epidermal autoregulatory factor 1 (Deaf-1, also known as NUDR) is one such protein[23]. It acts as a pre-synaptic repressor and post-synaptic enhancer of $5HT_{1A}$ expression: upregulating the synthesis and release of serotonin via its effect on pre-synaptic receptors, and increasing post-synaptic sensitivity to this monoamine[24]. A drug that leads to activation of Deaf-1 – or selective potentiation of its pre- or post-synaptic effects – would likely be an excellent antidepressant (see fig. 1).

## E. Deaf-1 and depression

Deaf-1 regulates expression of $5HT_{1A}$ by binding to a recognition site located in the $5HT_{1A}$ promoter region[23] via its SAND domain[25]. This site contains a functional single nucleotide polymorphism known as C(-1019)G[26]; when the G-allele is present, Deaf-1 is unable to regulate $5HT_{1A}$ promoter activity. Interestingly, the G-allele is associated with both depression and

[§] Unfortunately, the clinical utility of mirtazapine is limited by its major side-effects, which include a sufficient degree of sedation to impair driving ability when taken the night before[22].

suicide in humans, as well as resistance to antidepressant response. Furthermore, homozygosity for the G-allele is more strongly associated with completed suicide than is the heterozygous genotype. These observations support the conceptualization of Deaf-1 as an endogenous antidepressant, and underscore the viability of Deaf-1 as a therapeutic target for depression.

## F. GSK3β: a novel interactor of Deaf-1

Glycogen synthase kinase 3β (GSK3β) is a constitutively active serine/threonine kinase that phosphorylates Deaf-1 *in vitro*[27]. It is also an established target of two drugs used clinically in the treatment of affective disorders: lithium[28] and ketamine[29–31]. Lithium has been the gold-standard of treatment for bipolar depression for decades, and effectively manages both manic and depressive symptoms of this disorder[32]. While lithium has a diverse array of molecular effects, it is known to inhibit GSK3β at therapeutic concentrations, and this is widely believed to be an important component of its mechanism of action. Ketamine is an NMDA-receptor antagonist that leads to downstream inhibition of GSK3β *in vivo*, and possesses remarkably rapid antidepressant[5]. The mechanism of action of ketamine is also poorly understood, but both lithium and the specific GSK3 inhibitor SB-216763 have been shown to potentiate and sustain the antidepressant-like behavioural effects of ketamine in animal models[30,31]. Lastly, although acute effects of any drug are unlikely to result from changes in gene transcription, it is worth noting that another specific inhibitor of GSK3, AR-014418, has shown antidepressant-like effects in the forced-swim test[33]. Together, these observations point towards GSK3β as a possible target for the treatment of mood disorders.

## G. Purpose

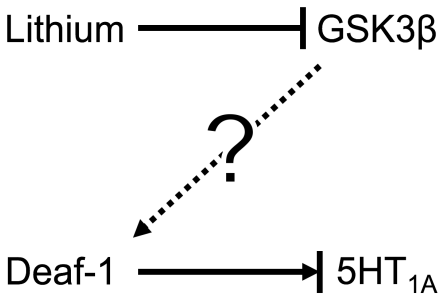


**Figure 2. Hypothesis.** Functional interactions at top and bottom are well established. We will attempt to link the two sets by manipulating different parts of the pathway.

$5HT_{1A}$, Deaf-1, and GSK3β each separately have clear importance in the treatment of depression. However, while the relationship between Deaf-1 and $5HT_{1A}$ is firmly established, it was not known whether GSK3β and Deaf-1 functionally interact. The present work tests the hypothesis that GSK3β regulates the activity of Deaf-1 and, by proxy, the $5HT_{1A}$ promoter by manipulating different parts of this putative pathway *in vitro* (see fig. 2). In order for our results to be readily translatable to *in vivo* experiments, GSK3β activity is manipulated exclusively using pharmacological methods. The effect of lithium in this model is of particular interest because the results of these experiments carry the possibility of shedding light on a piece of the mechanism of action of this important therapeutic. Using it and the specific GSK3 inhibitors CHIR-99021, SB-216763, and AR-014418 we conclusively demonstrate the existence of a functional GSK3β/Deaf-1/$5HT_{1A}$ pathway *in vitro*.

# Results

## A. HEK293 model of $5HT_{1A}$ promoter activity

When human embryonic kidney 293 (HEK293) cells were transfected with a reporter construct in which firefly luciferase expression was driven by the $5HT_{1A}$ promoter, co-transfection with a vector that led to overexpression of Deaf-1 repressed luciferase activity by ~45% (corrected $P < 0.05$). There was a low level of leaky luciferase expression in this vector, but luciferase activity was overwhelmingly due to the effects of the $5HT_{1A}$ promoter (see fig. 3). More importantly, there was no repression when the $5HT_{1A}$ promoter was absent (corrected $P = 1$). This important control experiment shows that the HEK293 model accurately reflects the regulation of $5HT_{1A}$ promoter activity by Deaf-1 in serotonin neurons.

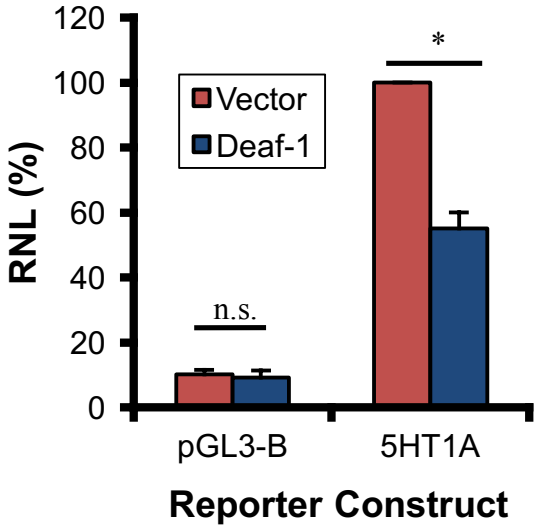


**Figure 3. Model of $5HT_{1A}$ promoter regulation in HEK293 cells.** Reporter construct refers to the pGL3-B firefly luciferase reporter with or without the $5HT_{1A}$ promoter. pcDNA3 was co-transfected with or without an insert containing the coding sequence of Deaf-1 (Deaf-1 and Vector, respectively). Units are relative normalised luminance (RNL), and error bars are standard error on the mean (SEM). * denotes corrected $P < 0.05$, n.s. denotes corrected $P > 0.05$.

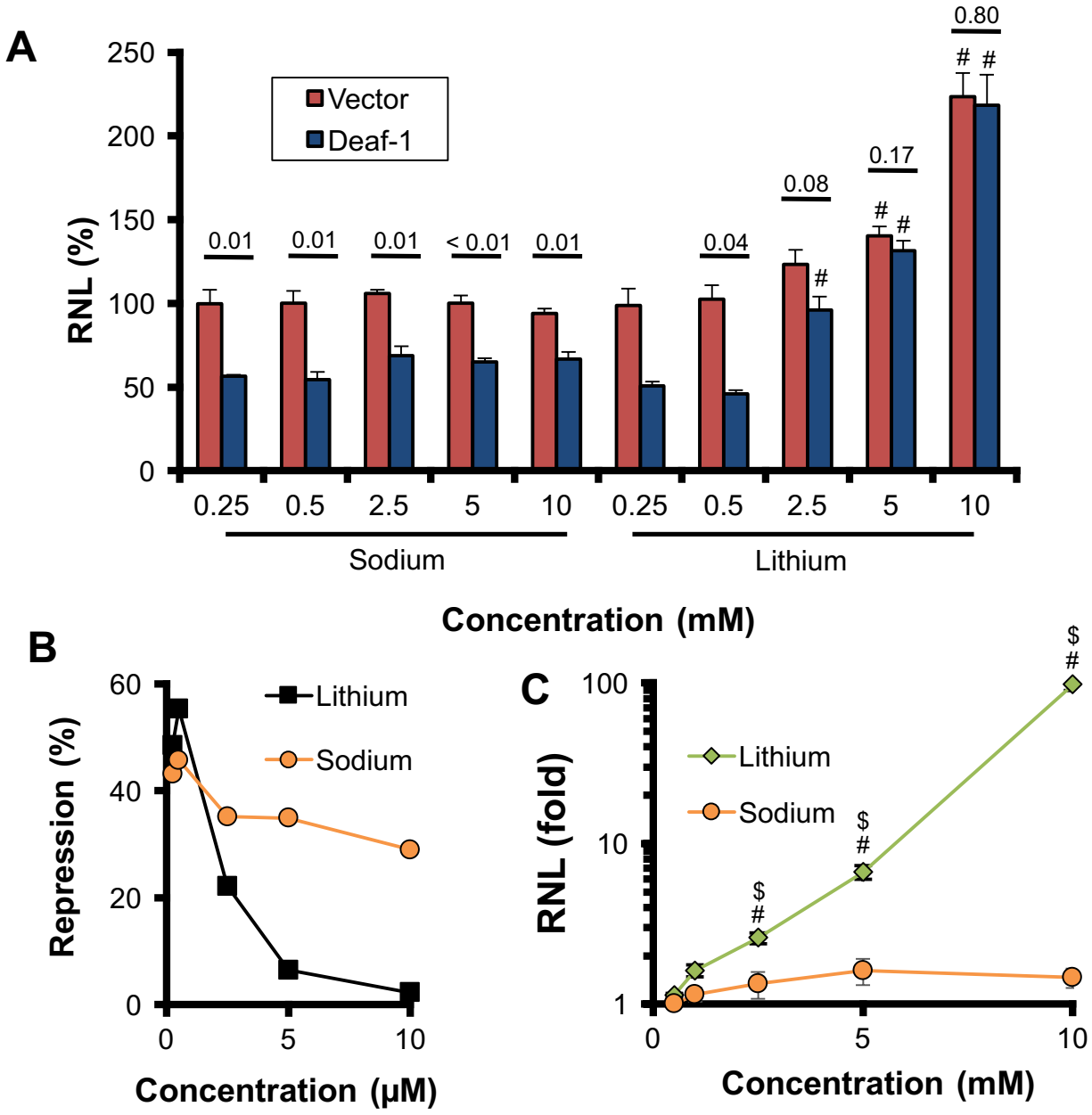


**Figure 4. Lithium assays.** All error bars represent SEM. **(A)** Effect of lithium on the $5HT_{1A}$ promoter in the presence and absence of overexpressed Deaf-1. Cation x plasmid x concentration interaction $P = 0.15$ ($F_{(1,91)} = 2.1$, $R^2 = 0.89$). *P*-values for repeated comparisons are presented uncorrected to examine trends in significance; one comparison was not presented because n = 2 in the Deaf-1 group. # denotes uncorrected $P < 0.05$ for comparison with the lowest concentration of the same Deaf-1 status. All other n = 4-6. **(B)** Concentration-dependent effect on percent repression by Deaf-1. Values are calculated based on means, and any confidence interval would be highly derived. This plot is therefore presented for ease of visualization, but is not analyzed. **(C)** Effect of lithium on GSK3β activity. Drugs were tested on cells transfected with a TCF/LEF firefly luciferase reporter, with higher RNL representing greater inhibition. Significant cation x concentration interaction ($F_{(1,26)} = 10.6$, $P < 0.01$, $R^2 = 0.65$). # denotes uncorrected $P < 0.05$ for comparison with the lowest concentration of the same drug. $ denotes uncorrected $P < 0.05$ for comparison between lithium and sodium at the same concentration. n = 3

## B. Lithium experiments in HEK293 cells

Lithium was chosen for our initial investigations into possible functional interactions of GSK3β and Deaf-1 because it is well-known to inhibit this kinase *in vivo*, and this is believed to contribute to its therapeutic effects in affective disorders. Lithium chloride was tested on the $5HT_{1A}$ construct in the presence and absence of overexpressed Deaf-1 at a range of concentrations (see fig. 4). Effects of altered osmotic pressure or extracellular chloride concentration were controlled for using a matched set of equimolar sodium chloride conditions. Initial analysis showed that lithium produced a concentration-dependent enhancement of $5HT_{1A}$ promoter activity that sodium did not[**] (concentration x cation interaction $F_{(1,91)} = 131.0$, $P < 0.001$). While Deaf-1 significantly repressed $5HT_{1A}$ activity in this experiment (main effect of plasmid; $F_{(1,91)} = 32.4$, $P < 0.001$), this effect was attenuated by high concentrations of either salt according to ANCOVA (concentration x plasmid interaction; $F_{(1,91)} = 10.8$, $P < 0.01$). The highest level ANCOVA interaction trended towards significance (concentration x plasmid x cation interaction; $F_{(1,91)} = 2.1$, $P = 0.15$). While this result strictly suggests that lithium did *not* attenuate Deaf-1 repression significantly differently than did sodium, the *post-hoc* analysis reveals a trend away from significant repression with increasing concentration of lithium, but not sodium (see fig. 4). Overall, the data in this experiment are consistent with two main trends: a concentration-dependent attenuation of Deaf-1 repression by lithium that is not fully accounted for by non-specific factors such as osmotic pressure, and a concentration-dependent enhancement of $5HT_{1A}$ promoter activity that is not tied to Deaf-1 overexpression.

[**] Note that, while typically only statistics from the ANCOVA are presented in the text, the *post-hoc* analysis is used to clarify how the ANCOVA should be interpreted. When possible, a synthesized interpretation of both analyses (along with the ANCOVA statistic) is described for the sake of brevity. See Materials and methods for more details on how data are presented and analyzed.

Lithium was tested in the GSK3β reporter construct as a positive control to determine whether any of the results from the previous experiments could be due to inhibition of this enzyme. In this assay, lithium concentration-dependently inhibited GSK3β and did so much more strongly than sodium (main effect of cation and cation x concentration interaction; $F_{(1,26)} = 28.7$, $P < 0.001$ and $F_{(1,26)} = 10.6$, $P < 0.01$, respectively). These results confirm that lithium concentration-dependently inhibits GSK3β in our conditions, and that this is not simply due to non-specific salt effects.

## C. Lithium experiments in MEF cells

Because lithium significantly enhanced $5HT_{1A}$ promoter activity independent of Deaf-1 overexpression, a follow-up experiment was conducted to determine whether this effect in the vector might be mediated by the endogenous Deaf-1 expressed by HEK293 cells. We used a line of embryonic fibroblast cells previously generated by our lab from Deaf-1 knockout mice (mouse embryonic fibroblasts; MEF) to test this possibility (see fig. 5). 10mM lithium significantly enhanced $5HT_{1A}$ promoter activity in cells transfected with the empty Deaf-1 vector condition compared to equimolar sodium chloride ($P < 0.01$), confirming that lithium causes Deaf-1 independent enhancement of the $5HT_{1A}$ promoter *in vitro*.

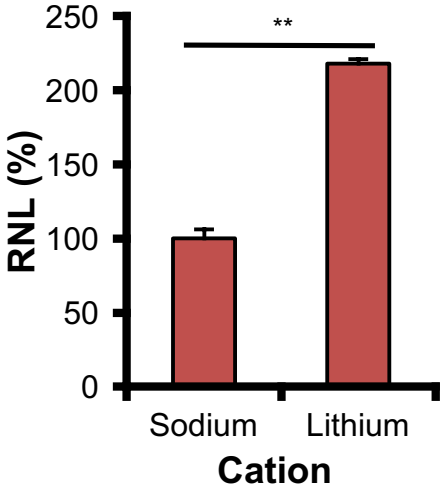


**Figure 5. Deaf-1-independent enhancer effect of lithium.** MEF cells that do not express Deaf-1 were transfected with the $5HT_{1A}$ promoter and empty Deaf-1 vector and treated with LiCl or NaCl at 10mM. ** denotes $P < 0.01$. n = 4

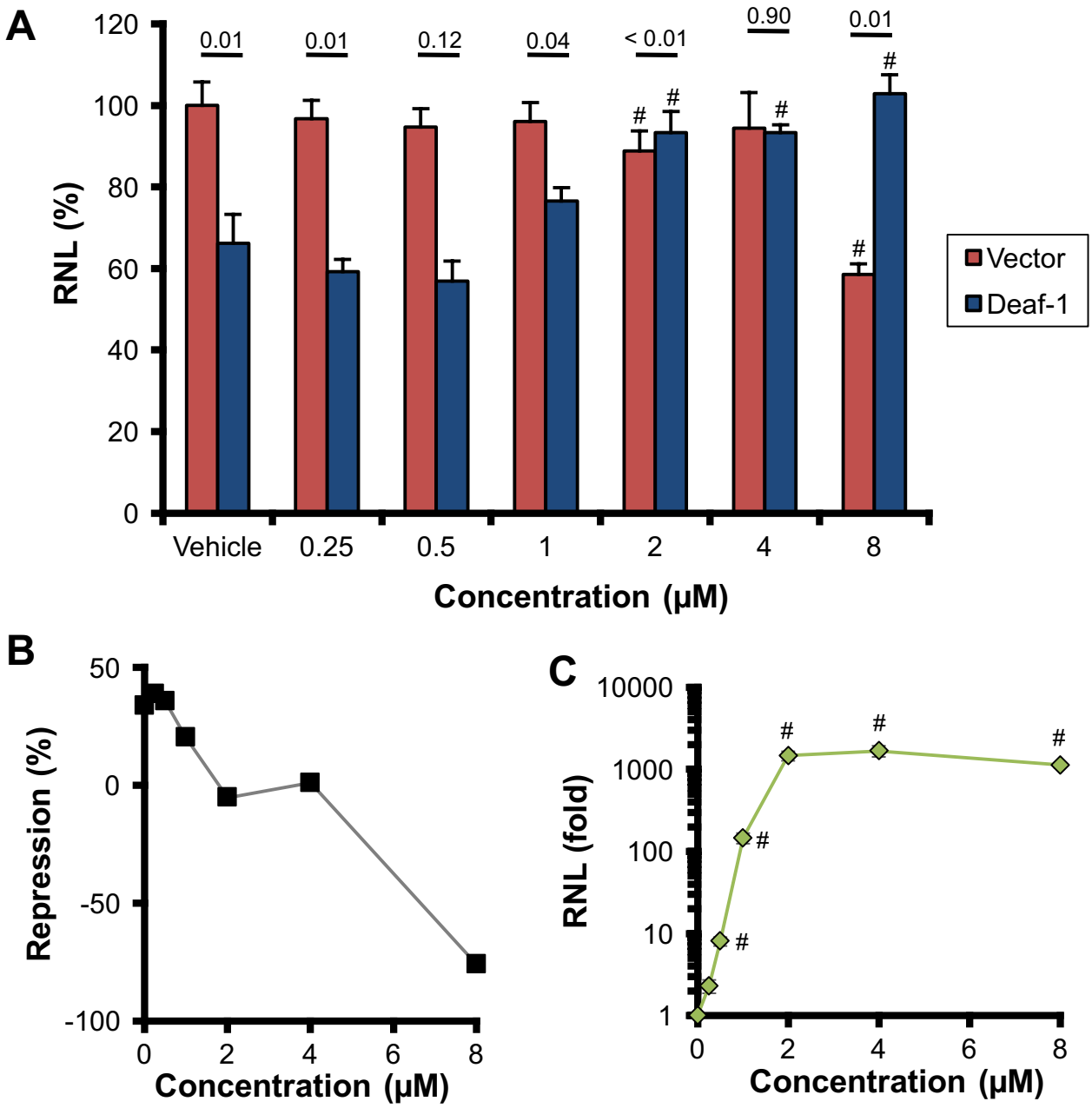


**Figure 6. CHIR assays.** All error bars represent SEM. **(A)** Effect of CHIR on the $5HT_{1A}$ promoter in the presence and absence of overexpressed Deaf-1. Significant concentration x plasmid interaction ($F_{(1,51)}$ = 73.6, $P < 0.001$, $R^2 = 0.63$). *P*-values for repeated comparisons are presented uncorrected to examine trends in significance. # denotes uncorrected $P < 0.05$ for comparison with vehicle of the same Deaf-1 status. n = 3-4. **(B)** Concentration-dependent effect on percent repression by Deaf-1. Values are calculated based on means, and any confidence interval would be highly derived. This plot is therefore presented for ease of visualization, but is not analyzed. **(C)** Effect of CHIR on GSK3β activity. CHIR was tested on cells transfected with a TCF/LEF firefly luciferase reporter, with higher RNL representing greater inhibition. Significant effect of concentration ($F_{(1,16)} = 49.6$, $P < 0.001$, $R^2 = 0.76$). # denotes uncorrected $P < 0.05$ for comparison with vehicle. n = 3

## D. CHIR-99021 experiments

Lithium had clear effects on Deaf-1 activity and GSK3β inhibition, but it is difficult to make a causal link between the two because lithium has many other targets. CHIR-99021 (CHIR) is a highly selective inhibitor of GSK3β that has previously been shown to potently inhibit this enzyme in HEK293 cells[34]. In our assay (see fig. 6), CHIR significantly attenuated the repression associated with overexpression of Deaf-1 (concentration x plasmid interaction; $F(1,51) = 73.6$, $P < 0.001$). Interestingly, CHIR also seemed to repress promoter activity at high doses, but only when Deaf-1 was not overexpressed, leading to an enhancer-like phenomenon in the repression analysis (see fig. 6a)

CHIR potently inhibited GSK3β (main effect of concentration; $F(1,16) = 49.6$, $P < 0.001$), an effect that was significant at all but the lowest concentration tested in the *post-hoc* ($P < 0.01$ in each case). Inhibition of GSK3β appeared to reach a maximum by the third highest tested dose (2μM), but it is important to note that GSK3β inhibition is confounded with TCF/LEF reporter activity in this assay. While it is theoretically possible that saturation of luciferase translation occurs *before* maximal GSK3β inhibition by CHIR, it will be assumed going forwards that the results of this assay fully reflect the effects of the drug.

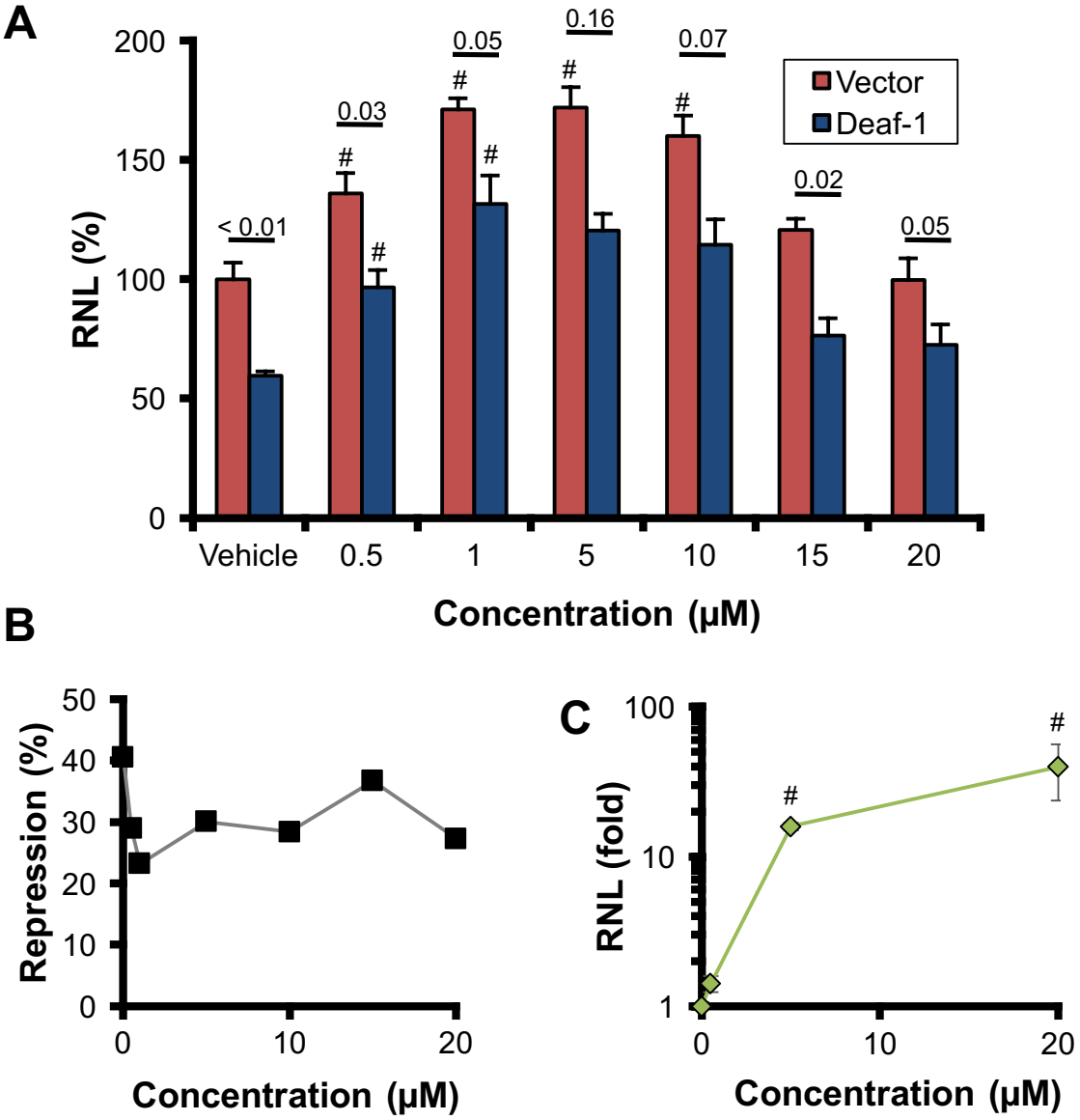


**Figure 7. SB assays.** All error bars represent SEM. **(A)** Effect of SB on the $5HT_{1A}$ promoter in the presence and absence of overexpressed Deaf-1. Significant concentration x plasmid interaction ($F_{(1,57)}$ = 0.50, $P = 0.48$, $R^2 = 0.40$). *P*-values for repeated comparisons are presented uncorrected to examine trends in significance. # denotes uncorrected $P < 0.05$ for comparison with vehicle of the same Deaf-1 status. n = 2-4. **(B)** Concentration-dependent effect on percent repression by Deaf-1. Values are calculated based on means, and any confidence interval would be highly derived. This plot is therefore presented for ease of visualization, but is not analyzed. **(C)** Effect of SB on GSK3β activity. SB was tested on cells transfected with a TCF/LEF firefly luciferase reporter, with higher RNL representing greater inhibition. Significant effect of concentration ($F_{(1,10)} = 25.0$, $P < 0.001$, $R^2 = 0.71$). # denotes uncorrected $P < 0.05$ for comparison with vehicle. n = 3

## E. SB-216763 experiments

SB-216763 (SB) is a specific inhibitor of GSK3β that is structurally dissimilar to CHIR[34]. While Deaf-1 repressed $5HT_{1A}$ promoter activity normally in these experiments (main effect of plasmid; $F_{(1,57)} = 22.1$, $P < 0.001$) (see fig. 7), no effect of SB was seen on extent of repression by overexpressed Deaf-1 (concentration x plasmid interaction; $F_{(1,57)} = 0.50$, $P = 0.48$). Because of the inverted U-shape of the effect of SB on overall $5HT_{1A}$ promoter activity (see fig. 7a), no concentration-dependent effect was seen in the ANCOVA (main effect of concentration; $F_{(1,57)} = 1.3$, $P = 0.26$). It is difficult to interpret the apparent loss of enhancement in both the Deaf-1 and empty-vector conditions at high concentrations, since this phenomenon was unique to SB in this project. However, it is interesting to note the enhancer activity that was independent of Deaf-1 status in other parts of the concentration range.

The GSK3β reporter construct was used to ascertain whether these results were due to an inability of SB to inhibit GSK3β in our conditions (e.g. due to poor solubility). However, a significant concentration-dependent effect was observed (main effect of concentration; $F_{(1,10)} = 25.0$, $P < 0.001$), indicating that this is not the case. Indeed, *post-hoc* analysis shows that SB clearly has a significant effect on GSK3β activity at concentrations as low at 5μM ($P < 0.001$); below the midpoint of our tested concentrations.

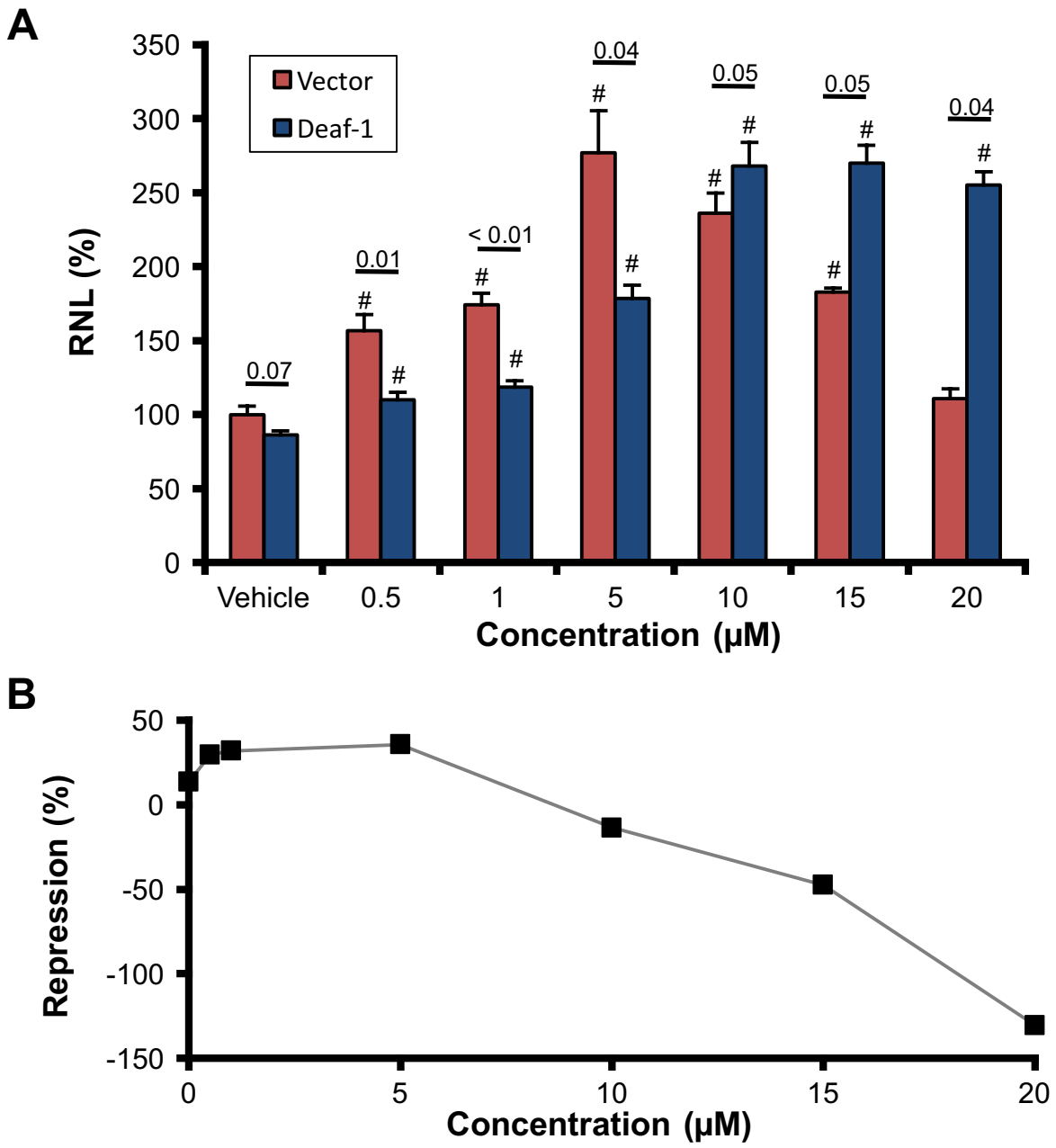


**Figure 8. AR-014418 assays.** All error bars represent SEM. **(A)** Effect of AR on the $5HT_{1A}$ promoter in the presence and absence of overexpressed Deaf-1. Significant concentration x plasmid interaction ($F_{(1,49)} = 20.2$, $P < 0.001$, $R^2 = 0.47$). *P*-values for repeated comparisons are presented uncorrected to examine trends in significance. # denotes uncorrected $P < 0.05$ for comparison with vehicle of the same Deaf-1 status. n = 3-4. **(B)** Concentration-dependent effect on percent repression by Deaf-1. Values are calculated based on means, and any confidence interval would be highly derived. This plot is therefore presented for ease of visualization, but is not analyzed.

## F. AR-014418 experiments

AR-014418 (AR) is a specific inhibitor of GSK3β that has been shown to have antidepressant-like effects in the forced-swim test[33,34]. In the $5HT_{1A}$ promoter assay (see fig. 8), AR enhanced promoter activity in both conditions (main effect of concentration; $F_{(1,49)} = 43.2$, $P < 0.001$) and also attenuated repression by Deaf-1 (concentration x plasmid interaction; $F_{(1,49)} = 20.2$, $P < 0.001$). Like CHIR, high concentrations of AR led to repression (or, equivalently, loss of enhancement) only in the vector condition.

AR was not tested further because of cytotoxicity issues with this compound, evidenced by drastically reduced cell viability at higher concentrations as well as abnormally low β-galactosidase activity compared to the DMSO vehicle (not shown). Additionally, the fact that the repression associated with Deaf-1 overexpression was relatively weak in the vehicle control places an important caveat on the results from this compound.

## G. Summary of important results

The present experiments tested lithium and three structurally-dissimilar GSK3 inhibitors in $5HT_{1A}$ and TCF/LEF promoter assays. All drugs except for SB led to a concentration-dependent decrease in the repressor activity of Deaf-1 on $5HT_{1A}$ promoter activity, which addresses the *a priori* hypothesis, but other trends were also observed. Firstly, all compounds except for CHIR concentration-dependently enhanced activity of this promoter in a manner that was not connected to Deaf-1 overexpression. In the case of lithium, the experiment in MEF cells showed conclusive evidence that this enhancement was Deaf-1-independent. Additionally, both CHIR and AR suppressed promoter activity only in the vector condition at high concentrations. These trends and the compounds with which they are associated are summarized in Table 1.

**Table 1. Summary of concentration-dependent statistics and trends from pharmacological assays.** Exact *P*-values are reported for the highest-level interaction of the ANCOVA, which tested the *a priori* hypothesis. Other results are based on a qualitative judgement of trends over multiple *P*-values, which are omitted here for the sake of brevity but can easily be found in the relevant figures.

| Compound | Attenuates Deaf-1 repression | | Other trends | |
|---|---|---|---|---|
| | ANCOVA | *Post-hoc* | Deaf-1-independent enhancement | Selective vector repression |
| Lithium | 0.15 | ✓ | ✓ | ✗ |
| CHIR-99021 | < 0.001 | ? | ✗ | ✓ |
| SB-216763 | 0.48 | ✗ | ✓ | ✗ |
| AR-014418 | < 0.001 | ✓ | ✓ | ✓ |

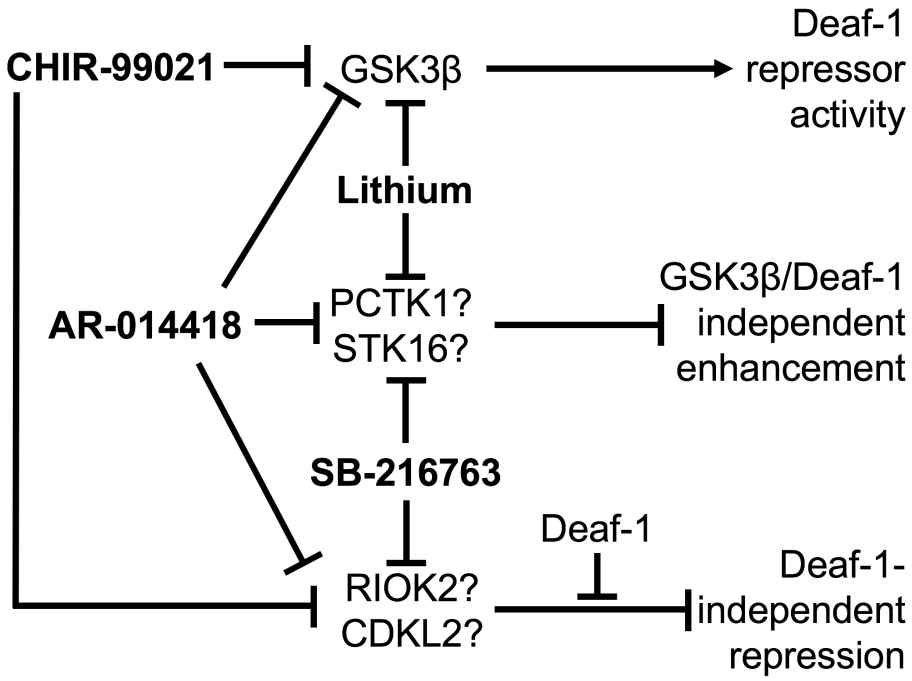


**Figure 9. Scheme of pathways that may account for the phenomena observed in pharmacological assays**. Proposed kinases are based on non-specific targets common to the relevant drugs; see Discussion for more information. Flat-ended connectors denote antagonistic activity.

# Discussion

## A. Interpretation of pharmacological assays

### Functional interaction of GSK3β and Deaf-1

The *in vitro* model accurately reflects the regulation of $5HT_{1A}$ promoter activity by Deaf-1, and under these conditions lithium and two selective GSK3 inhibitors concentration-dependently attenuated the repressor activity of this transcription factor. While the results of the ANCOVA analysis were not unequivocal in the case of lithium ($P = 0.15$), the linear model for this compound was significantly more complex than for the other drugs. In contrast, the *post-hoc* analysis showed a clear concentration-dependent attenuation of Deaf-1 repression by lithium that was either not present or much smaller in the sodium control. Because it becomes increasingly difficult to detect small effects with each added layer of model complexity using ANCOVA, it is likely that the slightly larger sample size in the lithium experiment was not sufficient to compensate for the drop in statistical power associated with an additional factor and two interaction terms in the model.

Overall, these results are consistent with the existence of our hypothesized GSK3β/Deaf-1/$5HT_{1A}$ pathway, except that SB did not show any evidence of affecting the extent of Deaf-1 repression. This negative result is particularly surprising because SB has been reported to be a more potent inhibitor of GSK3β than AR in HEK293 cells[34], but AR and not SB affected Deaf-1 repression.

However, it is important not to conflate potency with maximal efficacy. Under our conditions, SB approached maximal GSK3β inhibition at 5μM: well below the highest tested concentration of 20μM for this compound. The RNL of SB at 5μM is approximately one order of magnitude lower than that of CHIR at 1μM (the lowest concentration at which an effect on Deaf-1 repression is seen with this compound, see figs. 6,7). While such a comparison is hardly quantitative because of possible variations in the basal GSK3β activity – to which luminance was normalised – between experiments, this or other experimental factors are unlikely to account for a difference this large.

The GSK3β reporter used here contains *six* TCF/LEF binding sites, and is therefore highly sensitive to GSK3β activity. It is likely that GSK3β must reach a certain threshold of inhibition before any effects on $5HT_{1A}$ promoter activity are seen in our model, and that SB did not reach this threshold. Overall, our results support the hypothesis that Deaf-1 activity is regulated by GSK3β, and suggest that AR or CHIR may be better suited to future use in *in vivo* experiments.

**GSK3β-independent effects of GSK3β inhibitors**

Lithium enhanced $5HT_{1A}$ promoter activity through a mechanism that our MEF experiment showed was Deaf-1-independent. Similarly, both AR and SB produced some enhancement that was not clearly tied to Deaf-1 status. Inhibition of GSK3β precipitating increased $5HT_{1A}$ promoter activity through a Deaf-1-independent mechanism is not possible, however, because CHIR caused no such enhancement even when maximal GSK3β inhibition was achieved. This issue draws attention to a key limitation of this and all other pharmacological assays: no drug is 100% selective for a single target[††].

[††] This is also in large part why AR, SB, and CHIR were chosen for this project. The three are structurally dissimilar, meaning that their non-specific targets (i.e., those other than GSK3β) are less likely to be shared.

Occam's razor would dictate that this effect is mediated by some molecular target that is shared by lithium, AR, and SB, but not CHIR. Fortunately, the latter three have already been screened in parallel against a panel of 359 kinases[34], three of which fulfil these criteria: MST2, PCTK1, and STK16 (see Appendix). Because lithium inhibits MST2 by only ~6% at 10mM *in vitro*[35], the latter two kinases are the most likely candidates to explain the GSK3β/Deaf-1-independent enhancer activity seen in our results.

**Vector repression**

Another phenomenon unaccounted for by the GSK3β/Deaf-1 pathway is repression of the empty Deaf-1 vector at high concentrations of AR and CHIR. The possible contribution of GSK3β to this effect is again ruled out because it occurs at concentrations well above those sufficient for maximal inhibition with CHIR.

The most parsimonious interpretation of these observations is that Deaf-1 overexpression is inhibiting some other repressor, which itself is triggered by high concentrations of CHIR and AR (but not SB or lithium). In order for this to be the case, there would have to be a repressor that overexpression of Deaf-1 could somehow block, and CHIR and AR would have to share some way of triggering this repression that SB and lithium lack. Because the binding site of Deaf-1 in the $5HT_{1A}$ promoter overlaps with that of the repressor Hes5[26], it seems possible that overexpressed Deaf-1 could competitively inhibit Hes5 binding and, by proxy, repression. Endogenous Deaf-1, in contrast, might not be present at sufficient concentrations to outcompete Hes5 induced by the drugs. Based on the results of the same kinase screening discussed above, RIOK2, CDKL2, and BRAF(V600E) are three enzymes inhibited by CHIR and AR but not SB (see Appendix) that could account for induction of Hes5.

The main alternative explanation for the enhancer-like effect of overexpressed Deaf-1 is improbably intricate. If Deaf-1 is truly acting as an enhancer, then whatever mechanism is triggering this effect must be doing so with precisely the concentration-dependence to maintain RNL in the overexpressed Deaf-1 group at a consistent level that happens to coincide with the maximal RNL of the vector control. To add a layer of complexity, both AR and CHIR would have to share this exact property. Even the above were true, this hypothesis offers no explanation as to why RNL decreases at high concentrations of both compounds.

**GSK3β may activate the repressor activity of Deaf-1**

The model proposed above – in which Deaf-1 acts as a sort of transcriptional antagonist – has one important implication. If the effect of overexpressed Deaf-1 is neutral when GSK3β is inhibited, this would mean that GSK3β is specifically responsible for activating the repressor activity of Deaf-1, perhaps allowing it to recruit protein complexes that mediate the repression once it is bound to DNA. This hypothesis cannot be proven or rejected based on the data presented in the present work. However, now that a functional interaction between GSK3β and Deaf-1 has been conclusively demonstrated, it is useful to consider what the precise nature of this interaction might be[‡‡], and what those possibilities imply about the mechanism of action of lithium.

[‡‡] The other major possibility being that GSK3β phosphorylation is necessary for DNA binding.

## B. Implications for the mechanism of lithium

### Therapeutic relevance: connection to $5HT_{1A}$ expression in bipolar depression

Because our range of tested lithium concentrations bracket the therapeutic range, it is possible to make some inferences about what might occur in the brain over the course of lithium pharmacotherapy. In clinical settings, lithium is prescribed such that plasma concentrations are tightly controlled to the therapeutic range of 0.5-1.5mM[36]; because plasma concentrations imperfectly but closely reflect cerebrospinal fluid concentrations in rats[37], this range is likely to reflect the actual concentration range experienced by human neurons. Based on our results (see figs. 3 and 4b), lithium is predicted to attenuate the activity of Deaf-1 by up to 27% in humans. It may also activate the GSK3β/Deaf-1 independent enhancer mechanism, if the pathway that accounts for this phenomenon is present in human neurons.

The stimulatory effect of lithium on $5HT_{1A}$ promoter activity at clinically-relevant concentrations described in this paper is partially consistent with the results of a positron-emission tomography study showing increased $5HT_{1A}$ expression following lithium treatment in humans[38]. Notably, increases in a variety of cortical regions were associated with improvement in Montgomery-Asberg Depression Rating Scale scores over the treatment period, underscoring the importance of $5HT_{1A}$ heteroreceptors in the treatment of depressive symptoms. The enhanced $5HT_{1A}$ promoter activity shown in our assay provides a mechanism through which lithium might produce this effect.

However, the aforementioned study also determined that the expression of presynaptic $5HT_{1A}$ receptors was not significantly altered by lithium[38]. In our HEK293 cells, Deaf-1 repressed $5HT_{1A}$ promoter activity: a presynaptic phenotype. The results presented here would

seem to suggest that presynaptic $5HT_{1A}$ expression should be increased by lithium via GSK3β/Deaf-1 dependent and independent mechanisms. It is possible that this does not occur because the increased presynaptic transcription of $5HT_{1A}$ does not necessarily equate to increased expression on the cell surface, which may be controlled by compensatory translational and post-translational mechanisms *in vivo*.

It is also possible that the lack of change in dorsal raphe $5HT_{1A}$ by Nugent *et al.* is a false negative due to their small sample size (n = 12 patients)[38]. If this is the case, their results still support the conclusion that lithium *disproportionately* enhances the expression of postsynaptic $5HT_{1A}$. In either case, it is interesting to consider whether the GSK3β/Deaf-1-dependent or -independent enhancer activity of lithium might account for this differential regulation.

**Possible mechanism for regional differences in regulation of $5HT_{1A}$ by lithium**

It may initially seem difficult to reconcile the GSK3β/Deaf-1/$5HT_{1A}$ pathway described herein with the differential effect of lithium on pre- and post-synaptic $5HT_{1A}$ seen *in vivo*. For the sake of argument, it is reasonable to assume that GSK3β is the main pharmacological target of lithium. Further suppose that lithium distributes homogenously throughout the brain, and inhibits GSK3β by a similar amount in different regions. Assume also that GSK3β is responsible for activating the repressor activity of Deaf-1, as suggested above, and that this is true throughout the brain. From these assumptions, it would apparently follow that the GSK3β/Deaf-1 pathway cannot account for the differential effect of lithium on $5HT_{1A}$ – even if basal GSK3β activity differs somewhat between brain regions[§§] – because lithium should relieve Deaf-1 inhibition to a similar extent throughout the brain.

[§§] Unless, of course, basal GSK3β activity is nearly zero in the dorsal raphe, and much higher in the cortex. This seems relatively unlikely, however.

However, a differential effect of lithium on the pre- and post-synaptic GSK3β/Deaf-1 pathways could be made possible by a distinctive property of GSK3β: its preference for primed substrates[28***]. As discussed above, our results suggest that a certain threshold of GSK3β inhibition must be reached before lithium, CHIR, or any other GSK3β inhibitor affects regulation of Deaf-1/$5HT_{1A}$. If Deaf-1 is less extensively primed in postsynaptic cells, this may mean that the inhibition threshold is lower. Specifically, if GSK3β favours primed substrates more heavily when it is partially inhibited, the extent to which GSK3β phosphorylates a given target is then dictated by the extent to which that target is primed. Therefore, the effect of lithium on Deaf-1 could vary between cell-types, even if the effect of the drug on GSK3β does not.

This model of differential regulation of $5HT_{1A}$ is consistent with clinical characteristics of lithium and the results presented in this manuscript on two important related points. The priming model predicts that there would be a narrow "Goldilocks zone" of lithium concentrations that lead to decreased phosphorylation of Deaf-1 by GSK3β in cortical regions, but allow GSK3β to remain sufficiently active to phosphorylate Deaf-1 more extensively in serotonin cells. The narrow therapeutic window of lithium means that the possibility of such a zone is not immediately ruled out. Furthermore, lithium is predicted to only begin to relieve Deaf-1 inhibition at therapeutic concentrations based on our data. Because therapeutic concentrations of lithium are therefore likely to be close to the GSK3β inhibition threshold, small variations in this threshold could realistically allow for lithium to partially attenuate the GSK3β/Deaf-1 pathway in some cell-types but not others.

*** That is to say, GSK3β more readily phosphorylates substrates that have already been phosphorylated, or "primed", by some other kinase.

## C. Conclusion

$5HT_{1A}$, Deaf-1, and GSK3β are each important in the treatment of mood disorders. For the first time, this project makes a direct and conclusive link between these three proteins, and shows that pharmacological inhibition of GSK3β leads to attenuation of the repressor activity of Deaf-1 at the $5HT_{1A}$ promoter. Plausible explanations for the other effects of GSK3β inhibitors on $5HT_{1A}$ promoter activity have been presented in terms of non-specific actions of the relevant compounds at high concentrations. Lastly, this document has speculated extensively on possible connections between our *in vitro* results and the therapeutic action of lithium. While such speculation obviously does not allow any conclusions to be drawn, it may prove useful in directing follow-up investigations that could lead to a more complete understanding of a revolutionary pharmaceutical. Most importantly, knowledge of the pharmacology of drugs used in the treatment of affective disorders makes possible the rational design of improved therapeutics.

# Materials and Methods

## A. DNA Constructs

A total of five plasmids were used for all experiments. The most important consisted of the $5HT_{1A}$ promoter inserted into pGL3-Basic (Promega) between the Kpn I and BssH II restriction sites. It contains the full 1128b.p. sequence of the promoter, and possesses the functional C-allele at the polymorphic Deaf-1 binding site. The generation of this construct is described by Lemonde *et al.*[39], and is referred to by them as -1128-luc.

The construct used to overexpress Deaf-1 was based on pcDNA3 (Invitrogen). The full sequence of the protein was inserted into the EcoR I restriction site; the generation of this construct is described by Lemonde *et al.*[40], and is referred to as NUDR. Subsequently, a histidine tag was inserted upstream of the Deaf-1 sequence in order to facilitate experiments performed prior to the current work. To account for possible effects of the pcDNA3 vector itself in our experiments, an empty pcDNA3 vector control group was included in all experiments in which Deaf-1 was used.

The TCF/LEF luciferase reporter construct (pGL 4.49 from Promega) was used to measure GSK3β activity. It uses six copies of the TCF/LEF binding site to drive expression of firefly luciferase. GSK3β phosphorylates β-catenin, targeting it for degradation. When GSK3β is inhibited, β-catenin accumulates and binds TCF/LEF sites to drive gene expression. Increased activity of this construct thus signifies increased *inhibition* of GSK3β.

Lastly, a vector coding for β-galactosidase was co-transfected with the other constructs in all experiments. In pCMV-β-gal (Clontech), expression of β-galactosidase is driven by a viral

promoter, leading to maximal and largely unregulated expression of the enzyme. As a result, the activity of β-galactosidase was used as a control for all factors that globally regulate protein expression (e.g. nutrient abundance and growth rate) as well as experimental sources of variance (e.g. transfection efficiency from well to well).

## B. Drugs and Chemicals

Lithium chloride was purchased from EMD, while CHIR-99021, SB-216763, and AR-014418 were all obtained from Cayman Chemicals. Lithium was administered as a 1M solution in water, while specific inhibitors were administered dissolved in DMSO. Because DMSO is cytotoxic, its final concentration was kept to 0.1%, which has been shown to have no effect on cell growth[41]. However, following pilot experiments, it was decided to test higher concentrations of SB and AR. Consequently, the 15μM and 20μM groups for these compounds contained 0.15% and 0.2% DMSO, respectively. To reflect this, the vehicle control group for both of these drugs contained the highest concentration of DMSO (0.2%). All drugs were administered at the time of transfection.

Other reagents were obtained from Fisher Scientific, EMD, or Sigma, except where noted.

## C. Cell lines and cell culture

Human embryonic kidney 293 (HEK293) cells are a continuous cell line commonly used in research because of their ease of culture and transfection. They are particularly useful in the field of neuroscience because they share many physiological features with neurons. They express the serotonin transporter, a variety of neurotransmitter receptors (including $5HT_{1D}$, $5HT_6$, $D_2$, and $GABA_{B1}$), neurofilaments, and produce serotonin[42]. This may indicate that HEK293 cells are in

fact transformed adrenal medullary cells, which are themselves essentially post-ganglionic neurons of the sympathetic nervous system[43].

Because HEK293 cells express Deaf-1, some experiments were carried out in a mouse embryonic fibroblast (MEF) cell line. MEF cells were derived from a line of Deaf-1 global knockout mice generated by Hahm *et al*.[44]. The collection and transformation of MEF cells were carried out by Tristan Philippe and Bryce Le François, respectively, according to as-yet-unpublished procedures. Briefly, fibroblasts from fetuses (age E13.5-E15.5) were harvested and subsequently immortalized using the pWP TS A58 lentiviral vector. The genotype of the lot of MEFs used in the experiments presented here was verified by polymerase chain reaction amplification of the Deaf-1 locus followed by agarose-gel electrophoresis.

All cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) 319-015-CL (containing 4.5g/l glucose, glutamine, and without sodium pyruvate) supplemented with an extra 10% of foetal bovine serum (all from Wisent). Because of the possibility of latent infection associated with relatively new lines of tissue-derived cells, medium used to grow MEFs also contained penicillin and streptomycin. HEK293 cells were grown without antibiotics. Cells were washed with HBBS+EDTA (*s.i.c.*) (118mM NaCl, 4.6mM KCl, 10mM glucose, 20mM HEPES, and 5mM ethylenediaminetetraacetic acid; pH 7.2) (2.5g/l trypsin was added for MEF cells) in order to facilitate their suspension when passaging.

## D. Transfection

HEK293 cells were transfected using the calcium-phosphate method. Cells were sown onto six well plates at a density of $1.5x10^5$ cells/well and transfected the next day. A solution of 0.25M $CaCl_2$ containing 2ng/µl of each plasmid was prepared. To this, an equal volume of 2X

HEPES-buffered saline (280mM NaCl, 50mM HEPES, and 1.5mM $NaH_2PO_4$; pH 7.1) was added drop-by-drop. 200µl of the resulting solution was added to each well of cells.

MEF cells were transfected using FastFect (Feldan) lipid transfection reagent. Transfections were carried out according to the manufacturer protocol, except that the DNA:Fast-Fect ratio was optimized to 1:2.7. As with HEK293 cells, MEFs were sown onto six well plates at a density of $1.5x10^5$ cells/well the day before transfection. 1µg of each plasmid was used per well.

With both cell types, the next day the medium was removed, HBBS+EDTA used to wash away any remaining transfection mix, and fresh medium added. Cells were harvested and assayed for luciferase activity one day later, as described below.

## E. Luciferase Assay

Two days after transfection, cells were washed with phosphate buffered saline (137mM NaCl, 2.7mM KCl, 10mM $Na_2HPO_4$, and 1.8mM $KH_2PO_4$; pH 7.4) and then gently agitated in 300µl of 1X Reporter Lysis Buffer (Promega) for approximately 20min. The partially lysed cells were harvested and put through two freeze-thaw cycles in order to ensure complete lysis. Lysate was then centrifuged at 13,500xg for 2min to separate out any particulate matter. The resulting supernatant was used in the luciferase and β-galactosidase assays.

Luciferase assays were carried out using 100µl aliquots of each sample loaded onto opaque, white 96-well plates. (Occasionally, lysate was diluted in order to keep measurements in the linear range of detection.) A Reporter Lysis Buffer blank was included in each run to account for possible light leakage. Luminance counts were obtained using a Victor 3V (Perkin Elmer) at room temperature according to the following protocol: an equal volume of luciferase buffer

(20mM tricine, 972µM $MgCO_3$ pentahydrate, 2.4mM $MgSO_4$, 90µM ethylenediaminetetraacetatic acid, 24.3mM dithiothreitol, 326µM coenzyme-A, and 527µM adenosine-5`-triphosphate) containing 5.46mM luciferin potassium salt (Life Technologies) substrate was injected, mixed for 2s, and the luminance read for the next 5s.

β-Galactosidase activity was assessed using room temperature assays in clear 96-well plates. Aliquots of lysate were loaded onto the plates along with a Reporter Lysis Buffer blank (to account for non-enzymatic degradation of the substrate), and diluted in water in order to keep measurements in the linear range. For HEK293 cells, 2µl aliquots were used, while for MEFs the volume was 10µL. Reactions were carried out in Z-buffer (43.2mM $Na_2HPO_4$, 28.8mM $NaH_2PO_4$, 7.2mM KCl, and 0.72mM $MgSO_4$) containing 0.53mM chlorophenol red-β-D-galactopyranoside (Calbiochem) substrate in a final volume of 125µl. Absorbance at λ=570nm was read using a SpectraMax M5 (Molecular Devices) immediately after the last reaction was initiated and again at intervals. Reactions were mixed for 5s before the initial reading, and for 2s before each subsequent reading.

Luciferase activity varies based on transfection and harvesting efficiency in addition to regulatory effects at the promoter of interest. To account for this, blanked luminance was normalized to the activity of β-galactosidase (which was maximally expressed in our assay) from the same sample. β-Galactosidase activity was quantified as the difference between the initial and endpoint absorbance. In HEK293 cells, the endpoint was at either 4 or 6min (in order to keep measurements in the linear range), and in MEF cells it was 30min. In all cases, the same endpoint was used for all samples in a given run.

Following normalization, data was transformed to reflect the percent or fold difference from the vehicle (or sodium)/vector control. Units are therefore either percent or fold Relative Normalised Luminance (RNL).

## F. Optimization of the luciferase assay

The luciferase assay was initially performed one replicate at a time, with each sample of lysate being loaded into both assays in triplicate. This was done in order to obtain more accurate measurements, but was found to be unnecessary and time-consuming because the measurement error was usually only ~1%.

Also, the β-galactosidase assay was performed with lysate diluted ~200X and incubated at 37˚C for 1-2h before taking an endpoint reading. This, too, was time-consuming, and less accurate than the optimized procedure because it does not account for initial variations in clarity of the bottom of the plate, or small differences in incubation time due to the time taken to load the samples.

The initial HEK293 experiment and lithium assay (for the $5HT_{1A}$ promoter) presented above were performed using this rather than the optimized protocol.

## G. Elimination of outliers

It was occasionally necessary, especially in early experiments, to remove outliers in the data that were generated due to human error. The percent error was not expected to vary substantially between groups, and was typically ~5%. When the percent error was above 10%, the data was examined by eye to determine whether a single sample might account for this error, and removed accordingly. In cases when a single obvious outlier was not found, values further

than one standard deviation from the mean were removed iteratively. In order to avoid biasing the data in a particular direction by this iterative process (since the mean shifts each time a value is removed), pairs of values were removed above and below the mean before the new mean and standard deviation were considered.

## H. Sample size

The final sample size of n = 4 per group in $5HT_{1A}$ promoter assay was chosen based on a power analysis using estimated parameters from early experiments. It was estimated that a sample size of 4 would provide 80% power to detect a significant repressor effect of Deaf-1 in the *post-hoc* repeated T-test analysis.

## I. Data Analysis

Primary statistical analysis was conducted in R. Data from pharmacological assays in HEK293 cells was fitted with linear models describing RNL (percent and log fold, for experiments using the $5HT_{1A}$ and TCF/LEF promoters, respectively) as a function of concentration, plasmid (i.e., Deaf-1 or empty vector), and cation (for experiments using lithium), as applicable. Quartile-quartile plots of the residuals were used to confirm the suitability of these models. Residuals were normally distributed except at extreme values, which was expected because of the sigmoid nature of classical dose-response relationships. While this means that the models do not fully represent the data outside of the linear range, this small and symmetrical minority was not removed in order to present a more comprehensive analysis. The $R^2$ value for each model is presented in the figure caption. Analyses of variance (ANOVA) using marginal sum of squares were conducted to test the contribution of each factor to the overall model.

Further analysis was conducted in Excel (Microsoft). Plots of the percent repression of Deaf-1 over the vector control are shown for each assay using the $5HT_{1A}$ promoter. Because confidence intervals for this data would be highly derived, these plots are only presented to give a qualitative appreciation of the overall trend, and are not analyzed further. Two-sided t-tests for paired samples were used as a *post-hoc* analysis to examine important trends. *P*-values from these tests are presented uncorrected because they do not test independent null-hypotheses[†††45]. Thus, this *post-hoc* analysis should be interpreted by examining trends in significance.

For the initial control experiment using the $5HT_{1A}$ promoter in HEK293 cells, analysis consisted of two Bonferroni comparisons (two-tailed, paired sample; Excel). The MEF experiment was analyzed by two-tailed, paired sample t-test.

The significance threshold was set to $\alpha = 0.05$ in all cases.

[†††] For example, the null hypothesis that overexpressing Deaf-1 does not affect RNL when lithium is present at 5mM is not fully independent of the corresponding null hypothesis when lithium is present at 10mM.

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# Appendix

**Table 2. Kinases differentially affected by specific inhibitors of GSK3β.** Drugs were screened at 10μM against a panel of 359 kinases by Pilot-Storck *et al.*[27].

| Phenomenon | Kinase | Activity remaining with (%) | | |
|---|---|---|---|---|
| | | **CHIR-99021** | **SB-216763** | **AR-014418** |
| Deaf-1-independent enhancement | MST2 | 100 | 9.9 | 3.4 |
| | PCTK1 | 39 | 0.2 | 15 |
| | STK16 | 91 | 29 | 28 |
| Vector repression | RIOK2 | 22 | 95 | 23 |
| | CDKL2 | 3.8 | 87 | 40 |
| | BRAF(V600E) | 49 | 78 | 49 |